The Role of the Nitric Oxide-cGMP Pathway in the Kainic Acid Rodent Model of Seizure

UNIVERSITY OF LIVERPOOL

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By

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LIST OF ABBREVIATIONS

Akt/PKB	protein kinase B
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate
AC	Associational Commissural pathway
ADNF	activity dependent neurotrophic factor
ADNP	activity dependent neuroprotective protein
AED's	anti-epileptic drugs
ATP	adenosine triphosphate
BDNF	brain derived neurotrophic factor
BrdU	5-bromo-2-deoxyuridine
Ca	calcium
CA	cornu ammonis
CCI	controlled cortical impact
cGMP	3',5'-cyclic guanosine monophosphate
CNS	central nervous system
CREB	cAMP response element binding
DCX	doublecortin
DG	dentate gyrus
DRG	dorsal root ganglia
DNA	deoxyribonucleic acid
DW	distilled water
EEA	excitatory amino acid
EC	entorhinal cortex
EDRF	endothelial derived relaxing factor
EEG	electroencephalogram
eNOS	endothelial nitric oxide synthase
ERK1/2	extracellular signal regulated kinase 1/2
GABA	gamma amino butyric acid
GCL	granular cell layer
GFAP	glial fibrillary acidic protein
GTP	guanosine-5'- triphosphate
iGluRs	ionotropic glutamate receptors
ILAE	International League Against Epilepsy
iNOS	inducible nitric oxide synthase
KA	Kainic acid
LEC	lateral entorhinal cortex
L-NAME	N^G-nitro-L-arginine methyl ester
LPP	lateral perforant path
LTP	long term potentiation
MEC	medial entorhinal cortex
MF	mossy fibre
mGluRs	metabotropic glutamate receptors
MPP	medial perforant path
NADPH	nicotinamide diphosphonucleotide
NAP	NAPVSIPQ

NeuN	neuronal nuclei
NGF	nerve growth factor
7-NI	7-nitroindazole
NMDA	N-methyl-D-aspartate
nNOS	neuronal nitric oxide synthase
NO	nitric oxide
NOS	nitric oxide synthase
ODQ	1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one
OML	outer molecular layer
PBS	phosphate buffered saline
PCD	programmed cell death
PDS	paroxysmal depolarisation shift
PFA	paraformaldehyde
PI3-kinase	phosphoinositide 3-kinase
PNS	peripheral nervous system
PP	perforant path
PSA-NCAM	polysialated neural cell adhesion molecule
RMS	rostral migratory stream
SC	Schaffer Collateral pathway
SCN	suprachiasmatic nucleus
SE	status epilepticus
SG	stratum granulosum
sGC	soluble guanylyl cyclase
SGZ	subgranular zone
SNO	S-nitrosylation
SNV	stearyl-norleucine-17 VIP
SO	stratum oriens
SOD	superoxide dismutase
SVZ	subventricular zone
SIC	slow inward currents
TBI	traumatic brain injury
TGF	transforming growth factor
TLE	temporal lobe epilepsy
TNF	tumour necrosis factor
Tuj-1	beta III tubulin
VEGF	vascular endothelial cell derived growth factor
VIP	Vasoactive Intestinal Peptide

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The Role of the Nitric Oxide-cGMP Pathway in the Kainic Acid Rodent Model of Seizure

Anna Siobhan Cosgrave

Epilepsy is a common neurological disorder characterized by the recurrence of unprovoked seizures. Investigating the cellular and molecular events following a seizure leads to a greater understanding in the mechanisms that potentiate subsequent seizures. Nitric oxide (NO) regulates the excitatory neurotransmitter, glutamate, through N-methyl-D-aspartate (NMDA) receptors suggesting a role for NO in regulating neuronal excitability. NO is a gaseous molecule produced from L-Arginine via nitric oxide synthase (NOS) and can cross the plasma membrane of neighbouring cells to activate soluble guanylyl cyclase (sGC) by binding to the haem group of the enzyme to form guanosine-3',5'-cyclic monophosphate (cGMP). This can then initiate physiological responses in the cell, primarily via the phosphorylation of proteins by protein kinases. NO can also directly influence cell signalling pathways by nitrosylating membrane bound and/or intracellular proteins and transcription factors.

Certain types of epilepsy such as temporal lobe epilepsy (TLE) are often associated with a distinct pattern of neurological damage, such as neuronal cell death in the hippocampus. The proteins vasoactive intestinal peptide (VIP) and activity dependent neuroprotective protein (ADNP) are neuroprotective both in vivo and in vitro and have also been shown to be involved in development and neurogenesis. To investigate the role of the NO-cGMP pathway in the regulation of these proteins following seizure the NOS inhibitor, N-nitro-L-arginine methyl ester (L-NAME, 50 mg/kg), the neuronal NOS inhibitor, 7-nitroindazole (7-NI, 50 mg/kg) and the sGC inhibitor, 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ, 10 mg/kg) were used in the kainic acid (KA, 10 mg/kg) rodent model of epilepsy. 5-bromo-2-deoxyuridine (BrdU), a thymidine analogue which is incorporated into newly dividing cells, was used to investigate the role of NO in the regulation of these proteins in neurogenesis following KA treatment. All experiments were carried out in accordance with UK Home Office regulations.

Region-specific NO/NO-cGMP regulation of ADNP and VIP expression was observed in the hippocampus and DG at basal levels and following KA treatment. RT-PCR/QPCR and immunostaining showed that following KA treatment there was significant decrease in ADNP expression by 3 days in the hippocampus compared to vehicle controls. This was reversed by NOS and sGC inhibition suggesting the regulation of ADNP in the hippocampus to be via the NO-cGMP pathway. However, in the DG, regulation of ADNP was directly via NO and this regulation of ADNP was localized further to cell layers within the DG. VIP expression in the DG appeared to be via the NO-cGMP pathway following seizure, as a significant increase in VIP^+ cells was observed with NO inihibition and KA treatment. NO also regulates VIP expression in the CA3 region, interestingly, by 3 days an inverse relationship showing an increase in VIP^+ and a decrease in $GFAP^+$ cells was observed following seizure suggesting NO is also involved in glial activation in this area following seizure. BrdU-

labelling experiments showed NO regulated precursor cell proliferation and that this involved ADNP both in vivo and in vitro.

CHAPTER 1

General Introduction

1.1 Overview of Introduction

The cellular and molecular processes following a seizure can lead to neurological damage and changes in the brains circuitry, developing the individuals propensity to have more seizures. Such events are characteristic of epilepsy, a complex neurological disorder, and as one of the oldest disorders known to man, an extensive sea of literature exists on the topic. To explain the background on which this study is based, I will review some general features of epilepsy and seizure-related disorders and the region of the brain, called the hippocampus, which is often seen to display distinct patterns of neurological changes in epileptic patients and animal models of epilepsy. Hence the different types of cell death and the role of glia in epilepsy will be discussed. Neuroprotection and a brief introduction to the proteins investigated in this study, activity dependent neuroprotective protein (ADNP) and vasoactive intestinal peptide (VIP), followed by neurogenesis will be discussed. Finally, the role of the gaseous molecule nitric oxide (NO) and its signal transduction pathway NO-cGMP will be reviewed.

1.2 Epilepsy

1.2.1 Epidemiology

The ancient Greek philosopher Hippocrates (460-377BC) was one of the first to propose the idea that epilepsy was a disease rather than a divine affliction, or indeed, a demonic possession! There are many seizure types and mechanisms by which the brain generates seizures. It is a common neurological disorder, affecting approximately 0.4-1% of the human population. Although this is the global mean, in fact, epilepsy is more prevalent in developing countries than in more developed areas of the world. In developing countries such as Colombia, Ecuador, India, Liberia, Nigeria and Panama it has been reported that more than 10 in a 1000 people have epilepsy compared to 50 in 100,000 in developed countries (WHO, 2001). This may be due to a higher risk of infectious diseases because of living conditions. There are also pre- and post-natal complications and malnutrition leading to permanent brain damage which may, in turn, lead to epilepsy. Epilepsy is associated with an increased mortality, maybe due to status epilepticus (SE), whereby seizures do not terminate after 5 minutes or occur continuously without time for recovery,

which if untreated can cause brain damage and damage to other organs (WHO, 2001). Sudden unexpected death in epilepsy, termed SUDEP, is uncommon and the cause is unknown. Some studies have suggested that a part of the brain that controls breathing may be affected during a seizure causing the person to stop breathing. Although epilepsy can often be controlled with anti-epileptic drugs (AED's), approximately one third of patients do not respond to AED's, so-called 'refractory epilepsy' because of inadequately controlled seizures (Curry and Kulling, 1998). The long term side effects of AED's, such as a higher risk of developing cancer and birth defects, are also becoming increasingly evident (Kaplan, 2004; Singh et al., 2005) which gives rise to the need to develop safer drugs and drugs for those patients who do not respond to current AED's or surgery. Consequently, it is important to understand the cellular and molecular mechanisms leading to the development of epilepsy, a process which has been termed 'epileptogenesis'.

1.3 Seizure Types and Classification

A seizure (or ictus) is defined as a single episode of neurologic dysfunction generated by the hyperexcitability of neurons and hypersynchrony of neural circuits. This hyper-excitable state may be due to; alterations in voltage-gated ion channels (Lerche et al., 2005), a decrease of inhibitory neurotransmission, usually facilitated by gamma amino butyric acid (GABA) or increased excitatory neurotransmission by glutamate (Noe & Manno, 2005). The period after a seizure is often referred to as the 'postictal' period and the interval between seizures is referred to as the 'interictal' period (or epileptiform). Epilepsy is the recurrence of unprovoked seizures, implying that there are permanent pathophysiological or structural changes in the brain which are supporting abnormal hypersynchronous neuronal firing. It should also be noted that the term 'epilepsy' does not pertain to a single disorder but rather a group of syndromes with different symptoms and etiologies. Such syndromes include; temporal lobe epilepsy (TLE), which involves the synaptic reorganization of limbic circuitry (Sutula et al., 1989), childhood absence epilepsy, which is due to a thalamocortical circuit malfunction (Blumenfeld & McCormick, 2000), benign familial neonatal convulsion, involving the mutation of potassium channels (Jentsch 2000; Cooper & Jan 2003) and Glut-1 deficiency, whereby a

lack of the glucose transporter protein can lead to epileptic encephalopathy (Klepper 2004).

Epilepsies are generally classified according to a number of criteria such as their initial etiology, semiology, the origin of the seizure in the brain, the syndrome and the event that triggers the seizure. The classification of epileptic seizures and syndromes is still a difficult practice for specialists in the field but is important because this has significant implications for treatment and prognosis. In 1970 the International League Against Epilepsy (ILAE) put forward the first proposal to distinguish seizures from epilepsies (Merlis, 1970) and since then classifications have been reviewed and up-dated (Seino 2006). From clinical and electroencephalographic (EEG) analysis, seizures can be broadly divided into generalized and partial seizures. An EEG is a record of electrical potential (voltage) differences at paired points of the brain and it is these EEG patterns which can be used to determine epileptiform activity. Neurons in chronic epileptiform foci undergo spontaneous long (100-200 μs) and high (10-15 mV) depolarisations, associated with spike activity. This depolarisation is called paroxysmal depolarisation shift (PDS) which has recently been associated with slow inward currents (SICs) recorded in hippocampal pyramidal neurons (Browne & Holmes, 2003; Tian et al., 2005), however, PDS is not always a hallmark of epilepiform activity. Generalized seizures begin with a widespread electrical discharge involving both hemispheres of the brain, whereas partial seizures begin with an electrical discharge in a specific region of the brain. Partial seizures can be subclassified as simple or complex and can be caused by brain injury, infection, stroke and tumour; however, in most cases the cause is unknown. Additionally, not all epilepsy syndromes are permanent and some are only apparent during childhood.

1.4 The Hippocampus

1.4.1 Anatomical Organisation of the Hippocampus

Temporal lobe epilepsy (TLE) is the most common type of epilepsy in humans and is associated with a very distinct pattern of neurological damage that is seen in many, but not in all patients: neuronal cell death in the hippocampus, accompanied by fibrillary

gliosis and sclerosis of the hippocampus (Honavar, 1997). The hippocampus is situated in the medial temporal lobe of the brain and forms part of the limbic system. Ramón y Cajal originally defined the regions of the hippocampus as regio inferior and regio superior; however, it was the terminology of Lorente de Nó that has come to define what we now know as the hippocampus. The hippocampal formation is composed of the dentate gyrus (DG); the subiculum and the hippocampus (or hippocampus proper), which is further subdivided into the cornu ammonis (CA)1, CA2 and CA3 (Fig 1.1). However, it is often accepted that the entorhinal cortex, presubiculum and parasubiculum are also included in the term 'hippocampal formation' (Amaral & Witter 1995). Their connectivity is largely unidirectional and the cells most susceptible to excitotoxity are the pyramidal neurons in the CA1 and CA3 regions of the hippocampus, while the CA2 is resistant. The neurons in the hilus of the dentate gyrus (also referred to as the CA4 region) can also be sensitive to excitotoxity and a large proportion of them degenerate (Lowenstein et al., 1992). The laminar organization of the hippocampus includes; the principal cell layer (more commonly termed the pyramidal cell layer), a relatively cell free layer located outside the pyramidal layer called the stratum oriens and distal to this is the fiber-containing alveus (Fig. 1.1). Specific to the CA3, is the stratum lucidum which is a narrow layer occupied by the mossy fibers projecting from the dentate gyrus. Superficial to this layer in the CA3 and immediately above the pyramidal cell layer in the CA2 and CA1, lies the stratum radiatum. This suprapyramidal region contains CA3 to CA3 associational connections and also CA3 to CA1 Schaffer collateral connections (Fig 1.2). The stratum lacunosum-moleculare is the most superficial portion of the hippocampus and it is here that the perforant pathway fibers from the entorhinal cortex terminate.

1.4.2 Cellular Organisation of the Hippocampus

The principal neuronal cell type of the hippocampus is the pyramidal cell. These have a basal dendritic tree, the length of which is relative to its position along the transverse axis of the CA3; i.e. those closest to the dentate gyrus have, on average, the smallest dendritic trees, whereas those in the distal part of the CA3 have larger dendritic trees. Other cell types of the hippocampus include a heterogenous population of basket cells, whose cell

bodies reside in the pyramidal cell layer. They also have dendritic trees, but few dendritic spines. Other nonpyramidal cells of the hippocampus include various interneuronal cell types which are immunoreactive for GABA (Ribak et al., 1978). These interneurons and their terminals can be subdivided according to their morphological characteristics, including the distribution of their axonal plexuses, and their neurochemical identity i.e. by their neuropeptide and calcium-binding protein content (Houser, 2007).

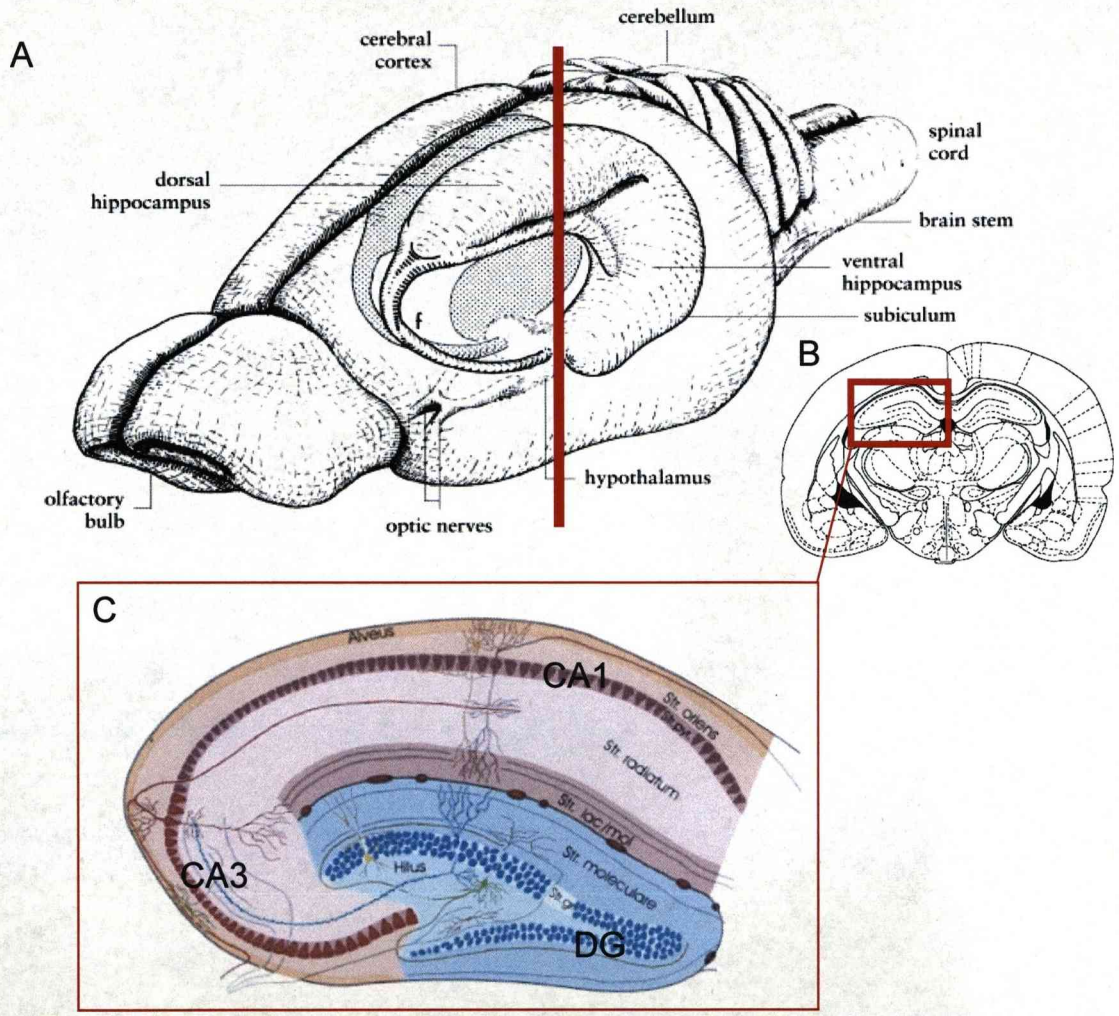


Figure 1.1 Schematic representation to show the three-dimensional organization of the rat hippocampus. A. Anatomy of the rat brain showing the C-shaped hippocampus in the septotemporal or longitudinal axis. B. Coronal cross section of the area represented by the red line in A. C. Magnification of the hippocampus highlighted to show the various cell layers. Adapted from Cheung & Cardinal, 2005 and http://www.uni-leipzig.de/~vetana/Hippocampus/hippocampus-farbig.jpg.

1.4.3 Pathways of the Hippocampus

The hippocampal circuitry is complex, its unidirectional connectivity is described in the context of a trisynaptic circuit, whereby afferent input from the entorhinal cortex *via* the perforant path synapses onto the dentate granule cells. The axons of the dentate granule cells (known as mossy fibres), in turn, project to the pyramidal cell layer (Fig 1.2). Initially, the mossy fibres synapse with the CA3 pyramidal cells, which then, *via* the Schaffer collaterals, synapse with the CA1 pyramidal cells and continue through the subiculum as efferent output to the cerebral cortex. However, it has recently been postulated that the CA3 also receives input from other pathways, suggesting the CA3 as another point of entry to the hippocampus rather than the DG. It is becoming increasingly evident that a 'backprojection' from the CA3 to the dentate gyrus exists and may be exerting its influence indirectly on granule cells via the GABAergic neurons (Scharfman, 2007). The main changes observed in the hippocampus which are associated with epileptogenesis include; selective neuronal loss, axonal and dendritic reorganisation, including 'mossy fibre sprouting' (Koyama and Ikegaya, 2004), dispersion of the dentate granule cell layer (Houser, 1990), changes in glial architecture (D'Ambrosio, 2004) and the altered expression of neurotransmitters and their receptors (Koyama and Ikegaya, 2005).

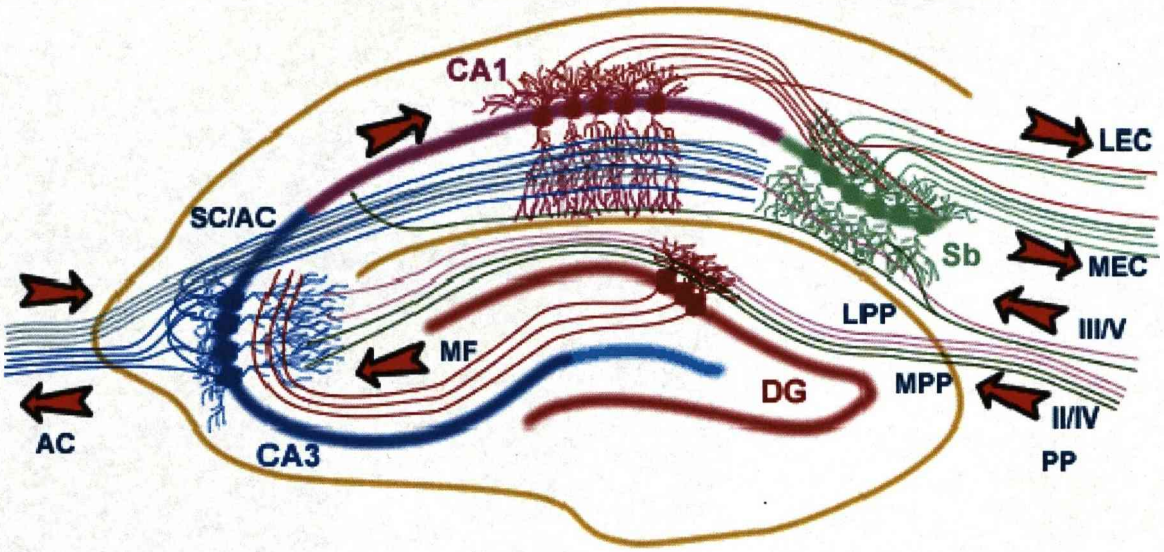


Figure 1.2 Pathways of the hippocampus. Afferents mainly arise from layers II and III, but also from deeper layers IV and V of the entorhinal cortex (EC-lateral entorhinal cortex, LEC; medial entorhinal cortex, MEC). Axons from layers II/IV synapse onto the granule cells of the dentate gyrus (DG) and CA3 pyramidal neurons via the perforant path (PP- lateral perforant path, LPP; medial perforant path, MPP). Mossy fibres (MF) of the DG also project to the CA3, the CA3 cells then project to the CA1 pyramidal cells via the Schaffer collateral pathway (SC) and to the contralateral CA1 via the Associational Commissural pathway (AC). Axons from layers III/V of the EC input directly onto the CA1 which projects to the subiculum (Sb) to output back to the EC. Taken from http://www.bris.ac.uk/Depts/Synaptic/info/pathway/figs/hippocampus.gif

1.4.4 The Dentate Gyrus

A three-dimensional view of the dentate gyrus shows an elongated curved structure folded within the hippocampus (Fig. 1.1). A transverse section through the dentate gyrus reveals a C or V shape, the dorsal limb referred to as the suprapyramidal blade (above the CA3) and the ventral limb referred to as the infrapyramidal blade (below the CA3). It is composed of three layers; i) a primarily cell free layer called the molecular layer (or stratum moleculare) which is mainly occupied by dendrites of the dentate granule cells and fibers of the perforant path that originate from the entorhinal cortex. A small number of interneurons are also found in the molecular layer along with fibers from other extrinsic inputs. ii) The principal cell layer of the dentate gyrus is known as the granule cell layer (or stratum granulosum) and is mainly composed of densely packed granule cells, however, other neuronal types can be found on the border between the granule cell layer and the hilus (or polymorphic layer) (Fig.1.1) e.g. the dentate pyramidal basket cell.

iii). The hilus, which is surrounded by the granule cell layer contains a number of cell types, mainly glutamatergic mossy cells and GABAergic interneurons. Distinct neuronal populations in the dentate gyrus, e.g. hilar neurons, display differential vulnerability to various forms of insult such as traumatic brain injury, ischaemia and epilepsy (Lowenstein et al., 1992; Jiao & Nadler, 2007).

1.5 Models of Seizure

In order to understand epilepsy and find new treatments, model systems must be employed. For this purpose, the use of animal models has disclosed much of what we have learnt about epilepsy and seizures. Animal models of epilepsy can include genetic abnormalities such as the WAG/Rij rat, alternatively, seizures can be induced via electrical stimulation e.g. the perforant path model or via chemical convulsants such as pilocarpine, bicuculline and kainic acid (KA) (Fisher, 1989). Kindling is a process by which the duration and behavioural involvement of induced seizures increases after seizures are induced repeatedly until a plateau is reached and so repeated stimulation 'lowers the threshold' for more seizures to occur (Racine, 1972).Kindling is usually carried out by focal electrical stimulation in the brain.The term 'seizures beget seizures' is often used to study this phenomenon, whereby a seizure may increase the likelihood that more seizures will occur. In the kindling model, seizures begin to occur spontaneously after repeated subconvulsive stimuli leading to self-sustaining status epilepticus (SSSE). However, the relevance of the kindling model in animals compared to epileptogenesis in humans is still widely debated and although there are a number of well-described anatomical changes associated with kindling (glial activation, decreased neuronal density, some mossy fiber sprouting), the changes are much less pronounced and widespread than those seen in epilepsy in humans (Cavazos and Sutula, 1990; Bertram, 2007).

1.5.1 Kainic Acid Model of Seizure

Kainic acid (KA) has long been established as a model for complex partial seizures (Nadler et al., 1978; Ben-Ari, 1985) and it induces an acute or subacute model of seizure which can last for hours or days. It is thought that subsequent hippocampal cell death

may confound the model. Alternatively, it can be used to investigate the pattern of limbic cell damage or the cellular and molecular changes leading to epileptogenesis following status epilepticus. Kainic acid is an excitatory amino acid (EEA) and is a cyclic analogue of the endogenous excitatory neurotransmitter, glutamate (McGreer et al, 1978) (Fig. 1.3). During the early 1950's a report by Hayashi described how the endogenous amino acids L-glutamate and L-aspartate induced tonic convulsion when topically applied to the motor cortex of the rat (Hayashi, 1954). This prompted the pioneering work of MacLennan, Curtis and Watkins who discovered that amino acids could have either excitatory or inhibitory effects on the central nervous system (CNS) and that there were distinct receptors for EAA's (Curtis et al., 1959; Curtis & Watkins 1965). EEA's are generally classified into ionotropic glutamate receptors (iGluRs) and metabotropic glutamate receptors (mGluRs) (Monaghan et al., 1989). Based on electrophysiological and pharmacological studies, iGluRs were further classified into three receptor classes named after their agonists, kainate, N-methyl-D-aspartate (NMDA) and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA). It is becoming increasingly evident that as well as exerting their ionotropic effects, kainate receptors expedite long-lasting signalling by novel metabotropic modes of action (Rodríguez-Moreno & Sihra, 2007). KA can activate NMDA and AMPA receptors as well as KA receptors and is well known for its excitotoxic effects (discussed later), but even at doses insufficient to cause cell death it can induce seizures in the hippocampus. At 4 mg/kg i.v. or 0.8-2.0 μg intra-hippocampally, animals can still display complex motor activity, masticatory movements, periodic arrest of activity and sometimes tonic-clonic activity (Lothman et al, 1981; Cavalheiro et al, 1982). For the purpose of animal models, the 'strength' of the seizure can be monitored according to the Racine scale (Racine, 1972), whereby the development of motor seizures is given a score from 1 to 5. These are as follows: (1) mouth and facial movements, (2) head nodding, (3) forelimb clonus, (4) rearing and (5) rearing and falling. The Racine scale is often used for various animal models of seizure including the KA model, the perforant path model, the pilocarpine and kindling model.

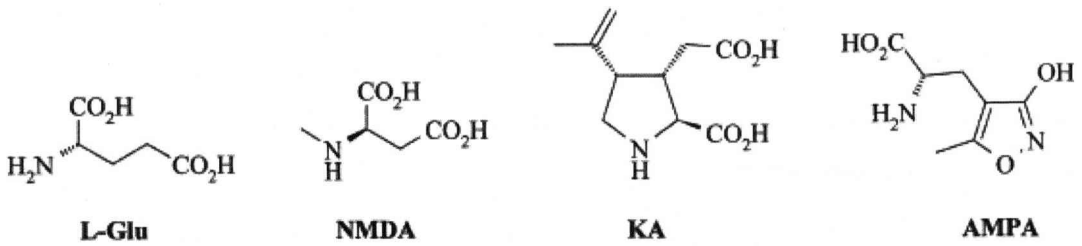


Figure 1.3 The structure of glutamic acid and its analogues N-methyl-D-aspartate (NMDA), kainic acid (KA) and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA). The nomenclature of these prototypic agonists is also given to the particular subtypes of excitatory amino acid receptors which they act upon.

1.5.2 Seizure Control by Diazepam

For ethical reasons it is often common practice to administer diazepam after inducing seizures with KA to reduce mortality and status epilepticus. Diazepam and related compounds are routinely used in the treatment of epilepsy and are members of the benzodiazepine family, the actions of which are to potentiate neural inhibition mediated by gamma-aminobutyric acid (GABA), the brain's main inhibitory neurotransmitter. This potentiation is confined to ionotropic $GABA_A$ receptors (as opposed to the metabotropic $GABA_B$ receptors) in the CNS to produce an increased chloride conductance. The $GABA_A$ receptor is formed by the assembly of multiple subunits (mainly α, β, γ, δ) into a pentamer. Benzodiazepines act allosterically via a specific site on the $GABA_A$ receptor independent from the GABA binding site to increase the affinity of the receptor for GABA. The presence of GABA to form this receptor-ion-channel complex then produces the main effects of sedation, hypnosis, decreased anxiety, anterograde amnesia, centrally mediated muscle relaxation and anti-convulsant activity. It has been shown that prolonged seizures or SE can lead to progressive alterations of $GABA_A$ receptors, including reduced surface expression of these receptors by receptor trafficking, which may be the cause of benzodiazepine pharmacoresistance in refractory epilepsy (Olsen & MacDonald, 2002; Jones et al., 2002; Loscher, 2007; Leidenheimer, 2008). Early discontinuation of SE with diazepam may reduce the intensity or delay the onset of recurrent spontaneous seizures and subsequently protect against hippocampal cell loss (Brandt et al., 2003; Pitkänen et al., 2005). Diazepam has also been shown to prevent the

seizure-induced increase in nitric oxide levels leading to lipid peroxidation which may subsequently cause neurodegeneration (Rajasekaran 2005).

1.6 Seizure Related Cell Death

The first reports of brain damage in epileptic patients go back as far as 1825 when Bouchet and Cazauvielh autopsied epileptic patients and reported elements of hippocampal sclerosis and hippocampal softening (Bouchet & Cazauvielh, 1825). Subsequently in 1880, Sommer went on to describe Ammon's horn (hippocampal) sclerosis (AHS) further. AHS is commonly seen in patients with TLE and is characterized by granule cell dispersion, reactive gliosis and a loss of pyramidal neurons in the CA1/CA3 and /or hilar neuronal cell loss (Margerison & Corsellis, 1966). Animal models of SE revealed a similar pattern of neuronal cell death (Meldrum et al., 1973; Olney et al., 1974; Ben-Ari, 1985).

How these cells die is not entirely understood, but it has been shown that both apoptosis and necrosis are involved in seizure-induced, region/cell-specific damage and that different cell death pathways are activated in different seizure models (Narkilahti et al., 2003; Fujikawa, 2005; Fujikawa et al., 2007). The main features of necrosis are ATP depletion and ionic disregulation leading to the swelling of mitochondria and endoplasmic reticulum. Subsequently, the entire cell swells, which eventually leads to the disruption of the plasma membrane and cell lysis (Saelens et al., 2005). Apoptosis is a form of programmed cell death (PCD). In the literature the terms PCD and apoptosis have been synonymous; however, not all kinds of genetic PCD are apoptotic in terms of the original definition. Apoptosis is a genetically regulated form of cell death, which plays an important role in tissue homeostasis, differentiation and development and is involved in many degenerative diseases, more relevantly, epilepsy. Typical morphological changes associated with apoptosis include plasma membrane blebbing, shrinkage of the cytoplasm, dilation of endoplasmic reticulum, nuclear chromatin condensation (pyknotic nuclei) and fragmentation into apoptotic bodies that are phagocytosed by neighboring cells. There are two apoptotic signal transduction pathways, the intrinsic pathway which is initiated through the release of cytochrome c from the intermembrane space of mitochondria, often

through the activation of proapoptotic members of the Bcl-2 family and the extrinsic pathway, which is receptor-mediated and involves the binding of death ligands such as hormones, growth factors and NO to cell surface receptors resulting in the recruitment of the adaptor molecule to the cytosol. NO may induce apoptosis by helping to dissipate the membrane potential of mitochondria, therefore making it more permeable for cytochrome c release to trigger apoptosis (Brüne, 2003). Whether a cell undergoes apoptosis or necrosis depends largely on the intracellular ATP concentration. It has been proposed that NO may alter ATP concentrations and the establishment of amplifying or inhibiting feedback loops that normally accelerate or prevent apoptotic cell death depends on the overall redox-state of the cell (Leist et al., 1999). Other types of cell death include autophagy, anoikis, Wallerian degeneration and excitoxicity. Following status epilepticus (SE), neuronal cell death is morphologically necrotic which is caused by the activation of intracellular proteases and neuronal nitric oxide synthase, with generation of free radicals, and damage to cellular membranes, structural proteins, and essential enzymes. Programmed cell death mechanisms, such as p53 activation, activation of cell death-promoting Bcl-2 family members, and endonuclease-induced DNA laddering, also occur in SE-induced neuronal cell death (Fujikawa, 2005).

1.6.1 Excitotoxicity

During some pathological conditions such as traumatic brain injury (TBI), stroke and epilepsy the resultant increase in glutamate causes the overactivation of NMDA, AMPA or KA receptors. The main mechanisms for glutamate-induced toxicity are post-synaptic Ca^{2+} influx leading to the activation of Ca^{2+} dependent intracellular signals and oxidative stress and free radical damage to DNA, lipids and proteins (Choi 1987). The neurotoxic effects of KA in the brain were primarily discovered when the stereotaxic injection of KA into the rat striatum was found to cause neuronal degeneration whilst sparing axons of passage, i.e. axons which pass through or terminate in the injected area (Coyle, 1978). KA induces seizures by causing the depolarization of cortical neurones via an increase in intracellular Ca^{2+} to trigger Ca^{2+}-dependent pathways. This excessive excitation can then lead to cell death either by apoptosis or necrosis in various regions of the brain (Fujikawa, 2005).

The excitotoxic effects of KA have been well documented. In particular, the CA1 and CA3 pyramidal neurons of the hippocampus show neurodegenerative characteristics, similar to the pathophysiology seen in the hippocampus of human TLE as previously discussed (Meldrum et al., 1973; Olney et al., 1974; Ben-Ari, 1985). It is still to be discerned how exactly KA exerts its toxic effects on the hippocampus even when injected systemically or into regions outside of the hippocampus (Olney et al, 1974). KA-induced selective vulnerability in the hippocampal neurons is thought to be related to the distribution and selective susceptibility of the AMPA/kainate receptors in the brain. It has been shown that the CA3 and CA1 show high expression levels of the kainate receptor genes for GluR6 and KA2 (Wisden & Seeburg 1993; Bureau et al., 1999). GluR6$^{-/-}$ mice where shown to be resistant to seizures by KA injection (Mulle et al, 1998). Also, using slice preparations from these GluR6$^{-/-}$ mice, it was shown that ablation of GluR6 prevents kainate-induced gamma oscillations or epileptiform bursts (Fisahn et al, 2004). The immediate early gene c-fos is thought to regulate the expression of GluR6 during KA-induced seizures in mice (Zhang et al 2002).

Other mechanisms via which KA may mediate its neurotoxic effects may be through the activation of intracellular proteases and nitric oxide synthase (NOS) and the generation of free radicals which can cause damage to cellular membranes, structural proteins, and essential enzymes. PCD mechanisms, such as p53 (a tumour suppressor gene) activation, activation of cell death-promoting Bcl-2 family members, and endonuclease-induced DNA laddering, occur in SE-induced neuronal death (Leist et al., 1999; Chipuk et al., 2004; Fujikawa, 2005). Glial-mediated inflammatory responses may also be involved in neurodegeneration (Myer et al., 2006).

1.7 Glia in Epilepsy

Glial cells (also called 'neuroglia' or simply 'glia') provide support for neurons and are important for development, synaptic plasticity and synaptogenesis (Christopherson et al., 2005) and play an important role in neuronal excitability and survival (Vezzani et al., 2008). The KA model of seizure can also be used to study the activation of glial cells and

inflammatory responses typically found in neurodegenerative diseases. Once merely considered the 'glue' in the nervous system, the role of glial cells in aberrant network excitability is being revised and is the subject of considerable interest yet again (Sun et al., 2006; Wetherington et al., 2008). Unlike neurons, which are thought to be post-mitotic, glia can undergo mitosis. The different types of glial cells in the CNS include microglia, astrocytes (also called astroglia), oligodendrocytes, ependymal cells (also termed ependymocytes), radial glia cells and NG2 cells (often referred to as oligodendrocyte progenitor cells). Radial glia cells play an important role as precursors during neurogenesis and it is now known that these cells can differentiate into neurons in the adult mouse brain (Merkle et al., 2004).

Astrocytic processes ensheath neurons and other glia to mediate glutamate reuptake, control interstitial volume and the ionic environment, notably potassium, and they can act as a component of the neurovascular system to regulate permeability of the blood brain-barrier (Ridet et al., 1997; Abbott et al., 2006). Astrocytes are activated (reactive) in response to many CNS pathologies, hence the term 'reactive gliosis', a reaction with specific structural and functional characteristics which has long been defined in the pathology of TLE. This process can involve the morphological and biochemical changes of pre-existing astrocytes but also the generation of new astrocytes from stem cells (Binder & Steinhäuser 2006; Ravizza et al., 2008; Borges et al., 2006). The inflammatory process involves the hypertrophy of astrocytic cellular processes to accentuate their stellate morphology and is associated with the upregulation of intermediate filament proteins such as glial fibrillary acidic protein (GFAP), vimentin, and nestin. Other proteins upregulated are mGluR3, mGluR5, mGluR8, NGF and its receptors; cytokines upregulated include TGFβ, TNFα, VEGF, interleukins 1,4,6 and 10, and the enzyme iNOS is also upregulated (Ridet et al., 1997; Lederboer et al., 2000). In contrast, glutamine synthetase, which converts glutamate to glutamine in astrocytes which is then cycled back to neurons to be converted back into glutamate is downregulated in sclerotic brain tissue removed during epilepsy surgery (Eid et al., 2004) The role of neuron-astrocytic-neuron signalling in abnormal neuronal synchronization in epilepsy may be due to these changes in expression or via other mechanisms such as; glutamate release

from astrocytes to modulate neuronal excitability (Jourdain et al., 2007), glutamate transporters via impaired glutamate uptake (Tanaka et al., 1997), water and potassium homeostasis leading to a reduction in extracellular space to cause hyperexcitability, possibly via aquaporin or potassium channels, AQP4 and Kir4.1, respectively (Dudek et al., 1990; Manley et al., 2000; Shang et al., 2005) and finally via gap junctions (Wallraff et al., 2006). It is not known whether reactive gliosis acts to oppose or exacerbate seizures. In a transgenic mouse model expressing a GFAP-herpes simplex virus-thymidine kinase transgene, reactive astrocyte ablation with the antiviral ganciclovir was found to potentiate neuronal degeneration and inflammation after controlled cortical impact (CCI), a model of traumatic brain injury (TBI) (Myer et al., 2006). Conversely, glia have previously been shown to be neuroprotective in both the CNS and PNS (Trendelenburg & Dirnagl 2005; Thippeswamy et al., 2005).

1.8 Neuroprotection

The prevention of neuronal cell death has been the 'holy grail' of many scientists for decades. Following seizure it is becoming increasingly apparent that neuroprotection involves the preservation of neuronal and network function (Sutula et al., 2003). The prevention of cell death does not necessarily prevent seizures (Milward et al., 1999), in contrast, some neuroprotective mechanisms may indeed promote seizures. The most effective course for neuroprotection in seizure is to arrest seizure activity, for example, treating status epilepticus in the premonitory phases can prevent seizure-related damage (Chen & Wasterlain 2006; Walker et al., 2002). Neuronal survival involves a number of signalling pathways such as the phosphoinositide 3-kinase (PI3-kinase)/protein kinase B (Akt) which inactivates proapoptotic proteins such as Bad and caspase-9 (Datta et al., 1997; Cardone et al., 1998) and the extracellular signal regulated kinase 1/2 (ERK1/2) pathways which may be activated by neurotrophins or Ca^{2+} influx via the NMDA receptor (Hetman & Gozdz, 2004). Thus, low level and chronic activation of NMDA receptors is associated with their neuroprotective affects, whereas sudden and excessive activation of extrasynaptic NMDA receptors are attributed to their neurotoxic effects. A similar dual role is seen with NO and is discussed later. BDNF and NGF, belong to a family of neurotrophic factors that promote cell survival, neurite outgrowth, phenotypic

maturation and synaptic functioning and have neuroprotective properties in vitro and in vivo (Shigeno et al., 1991; Guegan et al., 1998; Poo, 2001; Schabitz et al., 1997). It has been proposed that seizure-induced expression of neurotrophic genes may underlie the sprouting of the axons of dentate granule cells (mossy fibres) (Sutula et al., 1996). Endogenous neuroprotective mechanisms may also involve the glial trophic factor, vasoactive intestinal peptide (VIP), and its responsive gene product, activity dependent neuroprotective protein (ADNP).

1.9 Vasoactive Intestinal Peptide (VIP)

VIP is a widely distributed neuropeptide with neuroprotective and neurotrophic properties (Delgado et al., 2004; Gozes, 2002). Despite its name, 'vasoactive intestinal peptide' is also expressed in the brain as well as in the gastrointestinal tract, where its effects were first identified (Said & Mutt, 1970). VIP also acts as a neuromodulator, and is co-released from neurons with their principal neurotransmitter e.g. it is present in cholinergic neurons in the cortex and GABAergic interneurons in the hippocampus (Bayraktar et al., 1997; Sloviter et al., 1987). VIP has been implicated in epilepsy were it was shown to excite hippocampal CA1 and CA3 neurones (Phillis et al., 1978). It was also shown that chemically induced seizures result in the release of VIP from synapses in the hippocampus and other areas of the brain (Marksteiner et al., 1989). In the hippocampus, VIP is contained in the synapses of hilar interneurons which are resistant to seizure-induced damage (Sloviter, 1987). The regulation of VIP via the NO-cGMP pathway will be investigated in this thesis and will be discussed in more detail in Chapter 5.

1.10 Activity Dependent Neuroprotective Protein (ADNP)

The neuroprotective effects of VIP have lead to increasing interest to develop the peptide as a potential neuroprotective drug candidate, however, it subsequently became apparent that VIP-based drug design was hindered due to the instability and limited bioavailability of the peptide (Gozes et al., 2003). In order to exploit VIP as a potential therapeutic agent, potent neuroprotective lipophilic analogues of VIP were synthesized e.g. stearyl-norleucine-17 VIP (SNV). Also, potent neuroprotective peptide derivatives were

identified such as ADNP, activity dependent neurotrophic factor (ADNF) and shorter peptide derivatives such as ADNF-9, ADNF-14 and the eight amino acid NAP. NAP was subsequently shown to exert a protective effect on glia-depleted neurones in culture (Zemylak et al., 2000). Since the cloning of its gene (Bassan et al., 1999), ADNP has been shown to be an important player in embryogenesis, brain development, cancer and neuroprotection (Mandel et al., 2007; Pinhasov et al., 2003; Gozes et al., 2003). It may be predicted that, as an adaptive mechanism, neurons produce ADNP in response to seizure. This may be particularly true in areas where new neurons are being produced (neurogenesis), such as in the subgranular zone (SGZ). In view of this, the regulation of ADNP via the NO-cGMP pathway during basal conditions or following seizure and the interactions between NO-ADNP and neurogenesis are a primary focus of this work and so ADNP will be discussed in more detail in Chapters 3, 4 and 6.

1.11 Neurogenesis

Neurogenesis is the birth of new neurons in the adult mammalian brain. This phenonomenon only occurs in specific loci of the brain, these mainly being the subventricular zone (SVZ) and the SGZ of the dentate gyrus (Altman & Das, 1965; Kuhn et al., 1996). Neurogenesis occurs mainly in three stages; precursor proliferation, migration and differentiation, and integration and survival. The molecular mechanisms by which progenitor cells (or neural stem cells) differentiate and acquire their identity are just beginning to emerge. These neural stem cells are a subtype of radial glia-like astrocyte that expresses GFAP and nestin (Seri et al., 2001; Filipov et al., 2003). These proliferate, which gives rise to clusters of transit amplifying precursors that then divide further to generate neuroblasts. The identification of these SGZ precursors and neuroblasts is characterized by a sequence of marker expression as they mature (Kempermann et al., 2004) (Fig. 1.4). Progenitor cells are imunopositive for doublecortin (DCX) and polysialated neural cell adhesion molecule (PSA-NCAM) during proliferation and as early postmitotic neurons. After further differentiation they start to express calretinin (a calcium binding protein) and the transcription factors Prox-1, NeuN and Tuj-1. After subsequent differentiation into mature granule neurons, they become immunopositive for Prox-1, calbindin, NeuN and Tuj-1 (Fig. 1.4). In order to study

proliferating cells and hence, neurogenesis, a thymidine analogue called 5-bromo-2-deoxyuridine (BrdU) is often used. It is incorporated into the newly synthesized DNA of proliferating cells (during the S phase of the cell cycle) and consequently antibodies specific to BrdU can be used to detect proliferating cells. In order for the antibody to bind, this method of detection requires denaturating the DNA, by exposure to acid or heat. In the dentate gyrus, adult-born granule cells integrate into the existing hippocampal circuitry which may provide network plasticity necessary for learning and memory. It is thought that neurogenesis may play a role following brain injury or neurodegeneration. It has been found that prolonged seizures lead to an increase in the proliferation of granule cell progenitors in the dentate gyrus. This increased neurogenesis contributes to abberant network reorganization the hippocampus (Gray et al., 1998; Parent et al., 1997).

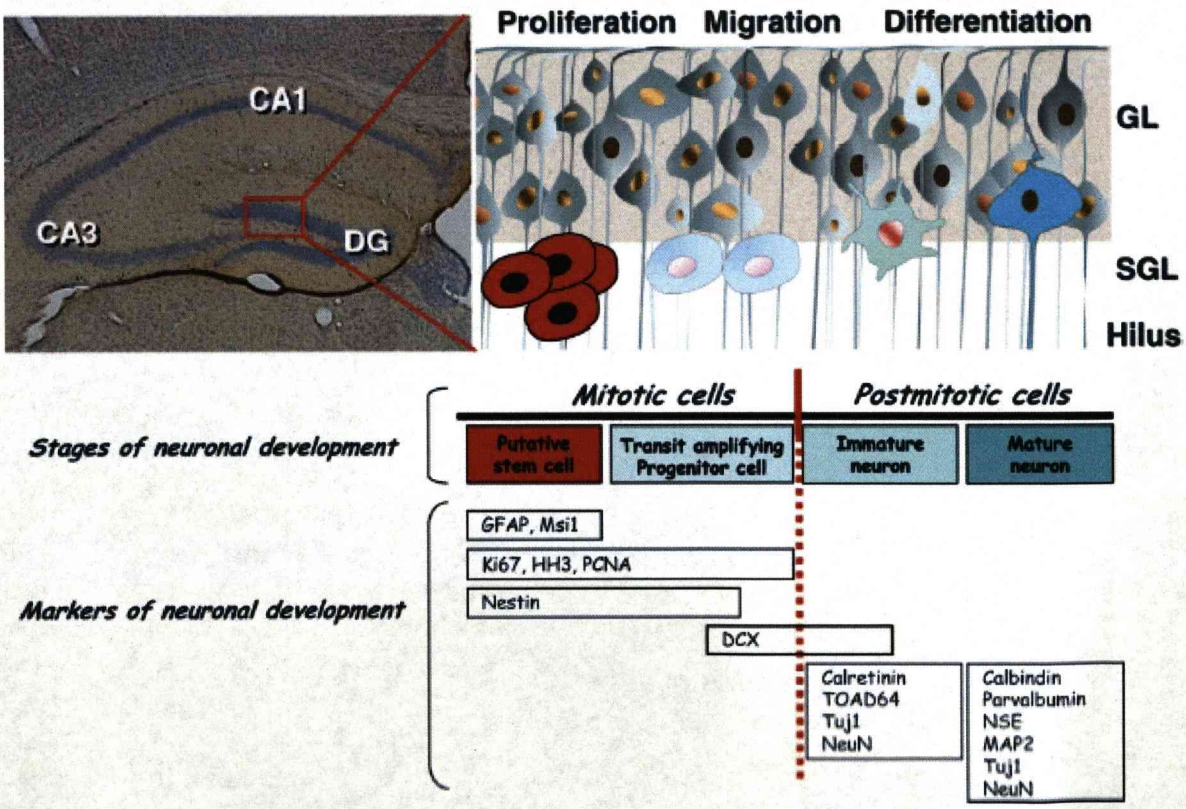


Figure 1.4 Hippocampal neurogenesis. Proliferation occurs in the subgranular cell layer/zone (SGL/SGZ) situated between the hilus and granular cell layer (GL), where cells then migrate and differentiate into mature neurons. The stages of neuronal development can be identified using specific cell markers (Abrous et al., 2005).

1.12 Nitric Oxide

Since the discovery that NO was, in fact, the molecule responsible for the effects of endothelial derived relaxing factor (EDRF) (Palmer et al, 1987; Garthwaite et al, 1988), the complex and intriguing biological activity of this free radical has been extensively studied over the years and is still the subject of much research. Generally, but not always, the direct effects of NO are attributed to low and brief NO production, supporting signaling and protective functions under normal conditions. Conversely, the indirect effects of NO are attributed to elevated and sustained NO production, consequently leading to toxic reactions such as oxidation, nitrosylation/nitrosation and nitration of proteins, such as those encountered during pathophysiological conditions such as; Alzheimers disease, multiple sclerosis, Parkinson's disease, epilepsy and stroke. The role and effects of NO vary with each biological system, cell type and the amount of NO within that system. Also, most of what we know about NO production in the brain has been obtained from the rodent model and may not always be applicable to other species.

NO is produced from its substrate, L-Arginine, by either; neuronal nitric oxide synthase (nNOS), inducible nitric oxide synthase (iNOS) or endothelial nitric oxide synthase (eNOS) depending on the cell type. NO can directly modulate proteins and ion-channels via s-nitrosylation and/or activate soluble guanylyl cyclase (sGC) to generate a second messenger molecule, cGMP *via* guanosine-5'- triphosphate (GTP).

1.12.1 Structure and Distribution of NOS

NO has 6 valence electrons from oxygen and 5 from nitrogen, and an unpaired electron in the last orbital confers its classification as a free radical. NO, along with citrulline, is produced from L-Arginine via NOS, as previously mentioned. This process requires the co-factor nicotinamide diphosphonucleotide (NADPH) and molecular oxygen. The structure of NOS has been well characterized and in its active form is often alluded to as a dimeric complex i.e. the binding of two NOS monomers. However, in this active state two calmodulin (CaM) molecules are also bound, in actual fact, making the active form of NOS a tetramer. Each isoform of NOS possesses flavin adenine dinucleotide (FAD),

flavin adenine mononucleotide (FMN), iron protoporphyrin IX (haem) and (6R)-5,6,7,8-tetrahydrobiopterin (BH4). These four prosthetic groups have specific roles vital for the activity of the enzyme with BH4 and haem constituting the substrate channel and FAD, FMN and haem all being involved in the redox reactions to produce NO itself (Wei et al., 2003; Mayer et al., 1991). The N-terminal domain of NOS has oxygenase activity with BH4, haem and L-Arginine binding sites. Meanwhile, at the C-terminal reductase domain, upon binding of calcium/calmodulin, this causes an influx of electrons to the active centre of NOS (Stuehr, 1999; Abu-Soud and Stuehr, 1993), thus activating the enzyme to generate NO from its substrate L-Arginine.

1.12.2 Nitric Oxide Synthase Isoforms and Splice Variants

NO is mainly produced from three NOS isoforms: neuronal NOS (nNOS, also referred to as Type I, NOS-I and NOS-1) and endothelial NOS (eNOS, also known as Type III, NOS-III and NOS-3), which are calcium/calmodulin-dependent enzymes and constitutively expressed in mammalian cells, and inducible NOS (iNOS, also known as Type II, NOS-II and NOS-2), which is calcium/calmodulin-independent and is synthesised *de novo* (Yun, 1997). In the nervous system, nNOS is present in neurons, astrocytes, and a subset of rat brain blood vessels (Law, 2001; Arbones, 1996; Nozaki, 1993). The eNOS isoform is present predominantly in endothelial cells and to a lesser extent, in astrocytes and neurons (Law, 2001; Colasanti; 1998; Dinerman, 1994). The iNOS isoform is inducible in cells of the immune system, and astrocytes, microglia and neurons (Law, 2001; Heneka, 2001). In addition to these well classified NOS isoforms, there are also spliced and post-translationally modified variants, such as nNOSβ, nNOSγ, nNOSμ and mitochondrial NOS (mtNOS), which is an isoform of nNOS found in the inner membrane of mitochondria (Bates et al., 1995, Elfering et al., 2002). Interestingly, both nNOSβ and nNOSγ lack PDZ domains and consequently do not bind PSD-95 from brain synaptic densities and do not associate with synaptic membranes (Brenman et al., 1996). Under normal physiological conditions, eNOS and nNOS produce NO in nanomolar concentrations for short periods in response to a transient increase in intracellular calcium. This is essential for normal brain function, such as neurotransmission, cerebral blood flow, synaptic plasticity, learning and memory,

neuroendocrine responses and behavioural activity such as stress and pain (Guix et al., 2005).

1.12.3 The Chemistry of NO

NO can undergo several chemical reactions to form nitrites (NO_2^-), nitrates (NO_3^-) and peroxynitrites ($ONOO^-$). Consequently, during nitrate production, other highly reactive intermediate products are also formed, such as hydroxyl radicals ($OH^\bullet$) and nitrite radicals ($^\bullet NO_2$) (Beckman and Koppenol, 1996). The peroxynitrite anion ($ONOO^-$) is formed due to the affinity of $O_2^{\bullet-}$ being greater for NO than for superoxide dismutase (SOD), an antioxidant enzyme (Cudd and Fridovich, 1982). Thus, NO may act as an oxidant whereby the effects of free radical oxidation of brain proteins leads to oxidative damage, subsequently, accelerating the aging process (Butterfield, 1997). However, other chemical reactions of NO such as protein nitration, nitrosylation/nitrosation and nitrotyrosination, play an important part in the physiology of the nervous system. Protein nitration involves the addition of a nitro group (NO_2) to the protein, generally with tyrosine residues. Although this is not the only form of protein nitration, one of the main molecules to nitrate proteins is peroxynitrite itself. It has been shown that leukocyte peroxidases can also nitrate proteins after the stimulation of immune cells (Eiserich et al., 1998; Brennan et al., 2002). The reaction of NO with the sulphur from a cysteine thiol leads to nitrosylation and nitrotyrosination. The nitration of active-site tyrosine residues is a post-translational modification which can alter protein structure and function and is produced in abundance during pathological conditions (Stamler et al., 1997; Tedeschi et al., 2007).

Despite being a free radical, NO has relatively weak reactivity and only interacts with other free radicals, oxygen and transition metals. Depending on the redox state of a cell, NO may also exist as a nitrosonium ion (NO^+), making it thermodynamically unstable (Stamler et al., 1992). Hence the chemistry of NO and its gaseous nature make it easy for NO to diffuse from producing cells into adjacent cells and act as a versatile biological messenger (Beckman et al., 1996), thus setting off the production of the classical second messenger pathway cGMP (Miki et al., 1977; Garthwaite et al., 1988).

1.12.4 Physiological Targets of NO

Since NO is a highly reactive and diffusible gaseous molecule it can function in an autocrine or a paracrine fashion. NO mediates its cellular signaling effects via several mechanisms, the main one being sGC leading to the formation of cGMP (Ignarro, 1991). The reaction of NO with the haem group of sGC gives rise to a conformational change, which in turn causes sGC to catalyze the conversion of GTP into cGMP. cGMP itself can then activate several downstream elements such as cGMP-dependent protein kinases e.g. PKGIα (where PKG isofrom expression can vary in dorsal root ganglia (DRG), spinal cord and brain regions), cGMP-regulated phosphodiesterases e.g. PDE5 and cyclic nucleotide-gated (CNG) cation channels (Denninger and Marletta, 1999) to mediate or modify neurotransmission and other cell functions such as smooth muscle relaxation or platelet aggregation. Excessive stimulation of the NMDA receptor subtype of glutamate receptors allows the influx of Ca^{2+} into neurones, which may augment NO production. The main intracellular action of NO is activation of sGC, but apart from increasing cGMP levels, NO also induces feedback inhibition of the NMDA receptor through a redox modulatory site on the receptor complex or via S-nitrosylation.

Cytochrome *c* oxidase is another protein target of NO which competes with oxygen during energy metabolism (Brown, 2001). The cytochrome *c* oxidase complex contains two haems and two copper centres. Binding of NO to this haem-copper centre inhibits cytochrome *c* oxidase (Cooper, 2002). However, this is only temporary inhibition, as NO readily dissociates from this complex, thereby causing the intermittent regulation of cytochrome *c* oxidase. Intermittent regulation of cytochrome *c* oxidase by NO is believed to regulate ATP production, oxygen consumption and possibly mitochondrial functions involved in neuroprotection or neurototoxicity (Beltran et al., 2000; Paxinou et al., 2001).

Other cellular signaling effects of NO include interaction with the mitochondrion (Clementi et al.,1998), oxidation and nitrosative stress, DNA and protein damage and S-nitrosylation (SNO) of intracellular and membrane bound proteins (PSNO). S-nitrosylation is the reaction of an NO group with cysteine residues in proteins, resulting

in an adduct called nitrosothiol. Previously, nitrosylation was largely overlooked as it was thought that the free radical NO lacked control and specificity to regulate signal transduction like other post-translational modifications such as phosphorylation. However, it is becoming increasingly evident that nitrosylation has more in common with phosphorylation than was originally thought such as reversibility, substrate specificity and spatio-temporal regulation. Due to the development of techniques such as the biotin switch assay, the detection of nitrosylated proteins has been made easier and hence the identification and characterization of nitrosylated proteins in recent years has escalated (Jaffrey & Synder (2001). S-nitrosylation of proteins can affect the function of many proteins including ion channels and nuclear regulatory proteins and the emerging roles of S-nitrosylation in protein misfolding and neurodegenerative diseases (Nakamura & Lipton, 2008) provide evidence that nitrosylation can regulate the overall physiology and pathophysiology of the nervous system.

1.12.5 NO in Epilepsy

Depending on the seizure stimulus, the cellular source of NO, and the activation of specific NOS isoforms, there is conflicting evidence regarding the role of NO either as an anti-convulsant (Penix et al., 1994, Kendrick et al., 1996; Gabriel et al., 2000; Sardo and Ferraro, 2007; Royes et al., 2007) or pro-convulsant (De Sarro et al., 1993; Tutka et al., 1996). The differing conclusions are mainly due to the complex interaction between the excitatory and inhibitory neurotransmitter pathways of the CNS. In the CNS, the release of glutamate by pre-synaptic neurons is regulated by GABAergic inhibitory input to presynaptic neurons. Glutamate binding activates NMDA receptors of postsynaptic neurons leading to the activation of calcium-calmodulin dependent nNOS to generate NO. It has been hypothesised that the downstream effects of NO modulate NMDA receptor (NR2B) function to prevent further calcium influx and/or suppress further glutamate release from the presynaptic neurons thus preventing seizures (Kalmer et al., 2005; Takata et al., 2005). The other hypothesis is that NO could also regulate GABAergic input to presynpatic neurons thus enabling NO to function indirectly as a pro-convulsant. iNOS as well as nNOS also appears to be increased during the acute phase of seizures (Murashima et al., 2002; Yang et al., 2003).

The role of NO in the cerebrovascular response to seizures has been elegantly demonstrated in the eNOS-/- and nNOS-/- mice in the kainic acid model, which has shown that NO is the mediator of all vascular changes. eNOS, either derived from endothelial cells or astrocytes, appears to mediate hyperaemia within the epileptic focus and together with nNOS participates in the maintenance of local cerebral blood flow in distant areas (Pereira de Vasconcelos et al., 2006). In addition, Chavko and colleagues have demonstrated that seizures produce a significant increase in nityrosine residues (the footprint of ONOO-) and that nNOS inhibition by 7-NI retards seizures (Chavko et al., 2003). Theses studies provide evidence that the NOS pathway is involved in seizure and also oxidative stress.

1.12.6 NO and Neurotransmitters

Pioneering techniques such as intracerebral microdialysis have allowed us to investigate neurochemical processes in the brain (Bito et al., Ungerstedt and Pycock, 1974). Due to the fact that measurements can be taken from discrete areas of the living brain, this has made possible direct comparisons of behavioural and neurochemical effects. Such studies have shown the modulation of NO/cGMP production by glutamatergic receptors in the cerebellum (Vallebuona and Raiteri, 1993), the cerebellum being abundant in glutamate receptors and glutamate mediated NOS/sGC production. Granule cells of the cerebellum contain high levels of NOS (Bredt and Synder 1990) as well as glutamate receptors NMDA, AMPA and metabotropic receptors (Nicoletti et al., 1986; Garthwaite and Brodbelt, 1989). The relationship between NO and neurotransmitters/neuropeptides, especially the brains major excitatory neurotransmitter, glutamate, is becoming increasingly evident. NO-mediated glutamate regulation via NMDA receptor activation can cause activation of nNOS, a process described in brain regions such as the hypothalamus, hippocampus, striatum and locus coeruleus (Prast 1996; Fedele et al., 2001; Trabace et al., 2004; Maura et al., 2000). This association of nNOS and the NMDA receptor forms a complex via the binding to the abundant postsynaptic density-95 protein (PSD-95) (Christopherson, 1999). It has been shown that NO enhances glutamate release in some brain regions and spinal cord and this regulation of glutamate by NO is thought

to be important for synaptic activation for long term potentiation (LTP) in learning and memory (Zorumski, 1998).

1.12.7 NO and Glutamate

Depending on the concentration of NO, its effects on glutamate transmission are biphasic, the most common example being during excitotoxity. Apoptosis, or programmed cell death, is an important process for neurophysiological homeostasis and initiation of the NO-cGMP pathway can lead to the activation of cGMP-dependent protein kinases and the inhibition of caspase activity (Dash et al., 2003). NO may inhibit apoptosis through cGMP dependent and cGMP independent mechanisms, such as that shown in stressed DRG neurons involving the suppression of bax, caspase-3 and -9 activation by NO (Thippeswamy et al., 2001). Activation of the NFκB pathway and the Jun-kinase pathways by NO can induce many genes in the regulation of neuroprotection and neurotoxity (Park et al.,1996; O'Neill and Kaltschmidt, 1997). A neuroprotective mechanism of NO, via feedback inhibition, has recently been demonstrated through Poly(ADP-ribose) polymerase (PARP), a zinc-finger DNA-binding "nick-sensing" enzyme, which is abundantly expressed by stressed neurons (Diaz-Hernandez et al., 2007). Upon binding to DNA, PARP becomes activated and cleaves NAD+ into nicotinamide and ADP-ribose. PARP then polymerises ADP-ribose on to nuclear receptor proteins including histones, transcription factors and PARP itself. It was recently shown that using PARP-1 knockdown by RNA interference relieved the inhibitory effects of NO on iNOS promoter activity. From biotin switch assays it was concluded that NO feedback inhibits iNOS gene transcription by S-nitrosylating the trans-activator PARP-1 and decreasing its binding and/or action at the iNOS promoter (Yu et al., 2006).

Stimulation of the NMDA receptor by glutamate via calcium influx to activate nNOS may exert a neuroprotective role via inhibition of caspases, conversely, sustained NMDA receptor activation leading to high concentrations of NO can cause neurotoxicity (Dash et al, 2003; Dawson et al., 1991). Interestingly, increased endogenous NO and/or calcium concentration as a result of sustained NMDA receptor activation can lead to autoinhibition of nNOS (Rogers and Ignarro, 1992) and/or S-nitrosylation of the NMDA

receptor to prevent neurotoxicity (Reveiwed Nelson et al., 2003). Recent studies on NMDA receptor sub-units have shown that NR2A, rather than NR2B, receptors predominantly co-immunoprecipitated with nNOS suggesting a more precise mechanism by which NMDA receptors may be interacting with nNOS (Al-Hallaq et al., 2007).

1.12.8 NO and GABA

As with glutamate, depending on the concentration of NO, its effects on GABA release are also biphasic. At basal levels NO can cause a decrease in GABA release, but at high concentrations NO can cause an increase in GABA release (Getting et al., 1996). GABAergic interneurons possess NOS and glutamate receptors (Chan Palay, 1982), however, Purkinje cells in the cerebellum have been found to express the NO target sGC, the cGMP dependent protein kinase and the G-substrate but not NOS, maybe suggesting a paracrine role for NO. Recent innovative experiments by Wang et al determined more accurate NO physiological concentrations in the nucleus tractus solitarii (NTS), a brainstem nucleus involved in processing central and sensory information, including cardiovascular function. Using rat brainstem slices they described that, while lower concentrations of NO may be important for fine tuning of glutamatergic transmission, higher concentrations are required to directly engage GABAergic inhibition in the NTS (Wang et al., 2007). GABA release by NO is thought to be mediated via two mechanisms; a Ca^{2+}-dependent release system and a Na^{+}-dependent carrier-mediated GABA uptake system (Ohkuma et al., 1996). In the hippocampus it has recently been shown that interneurones express sGC in their presynaptic GABAergic terminals, suggesting a role for retrograde NO signaling in these interneurones (Szabadits et al., 2007).

1.12.9 NO in Neurogenesis

The role of NO in neurogenesis is the subject of recent interest and it has been shown that NO reversibly inhibits cell division (Garg and Hassid, 1990) and that it inhibits cell proliferation in the adult mammalian brain (Packer et al., 2003). However, it has also been shown that induction of neurogenesis is attributed to the production of NO via iNOS or eNOS (Reif et al., 2004; Zhu et al., 2003). Recently, evidence for the role of NO in

neurogenesis in the adult rat dorsal root ganglion has emerged. NO was shown to regulate neural stem cell proliferation and differentiation both in vitro and in vivo which was correlated with the modulation of the transcription factor, neuron-restrictive silencer transcription factor (NRSF/REST) during nerve injury (Arora et al., 2007) . Trafficking of NOS within the cell is of great importance and recently identified proteins such as NOSTRIN (NOS traffic inducer) and NOSIP (NOS interacting protein) have been shown to regulate intracellular trafficking of eNOS and possibly other proteins in the plasma membrane which may be of importance for cell proliferation or apoptosis (Icking et al., 2005; Schleicher et al., 2005). The role of NO in neurogenesis, like most of its other functions, is complicated and how it regulates neurogenesis normally, may not be how it is regulating neurogenesis under pathological conditions. To this end, I have investigated the relationship of NO and ADNP in neurogenesis following seizure. Hence, the role of NO in neurogenesis and cell proliferation will be discussed further in Chapter 6.

1.12.10 Intervention of the NO-cGMP Pathway

The pharmaceutical industry contstantly endeavours to produce more potent and selective NO inhibitors in the hope of finding more specific therapeutic agents for disorders such as osteoarthritis, tumours and asthma. The selectivity of NOS ihibitors is of much debate with regard to what constitutes selectivity (fold difference), how it is defined (ratio of IC_{50} values or ratio of K_i values) and determined (the effects on isolated enzymes in cells or in vivo). Also, assays to screen NOS inhibitors have been carried out over short periods of time with no pre-incubation, which can lead to the underestimation of the potency and efficacy of slow onset inhibitors (Alderton et al., 2001). Many NOS inhibitors have been synthesized and these can be used as pharmacologial tools to study the role of nitric oxide. Some of the most widely used are L-NMMA, L-NNA and its methyl ester prodrug L-NAME (N^G-nitro-L-arginine methyl ester) (Fig. 1.5). These are non-selective NOS inhibitors and act to inhibit all forms of NOS. 7-NI has been described as an nNOS inhibitor however, its actions may not be highly selective but it has been extensively used in the absence of a more potent highly selective nNOS inhibitor. In the quest for highly selective inhibitors, the most progress has been made with iNOS with compounds like 1400W, GW273629 and GW274150 (Garvey et al., 1997; Alderton et

al., 2005). To investigate downstream 3',5'-cyclic guanosine monophosphate (cGMP) pathway of NO, the potent inhibitor of the soluble guanylyl cyclase (sGC), 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ), has been widely used.

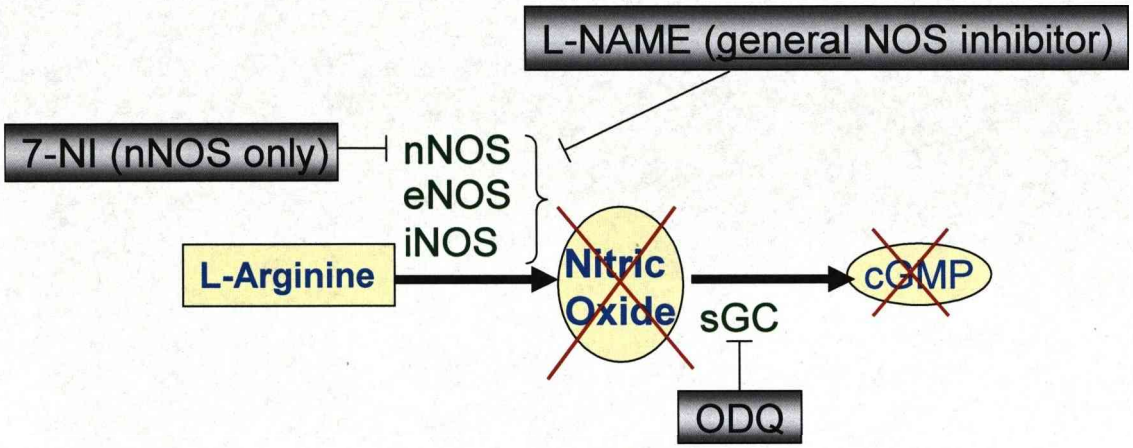


Figure 1.5 Intervention of the nitric oxide-3',5'-cyclic guanosine monophosphate (NO-cGMP) pathway with pharmacological inhibitors of nitric oxide synthase (NOS) and soluble guanylyl cyclase (sGC).

The relationship of NO in epilepsy and hippocampal cell death is not completely understood as it has been shown to have both anti-convulsant (Penix et al., 1994, Kendrick et al., 1996; Gabriel et al., 2000; Sardo and Ferraro, 2007; Royes et al., 2007) and pro-convulsant actions (De Sarro et al., 1993; Tutka et al., 1996), depending on the species studied and epilepsy model employed. Several factors may contribute to hippocampal cell death in epilepsy such as excessive gulatamate realease and activation of NMDA receptors leading to calcium-induced cell death. The calcium influx activates neuronal nitric oxide synthase (nNOS) to produce NO (Garthwaite, 2005). NO activates soluble guanylyl cyclase (sGC) to generate cGMP which can initiate physiological responses in the cell, primarily via the phosphorylation of proteins by protein kinases. NO can also directly modulate the expression of transcription factors, such as, CREB (a transcription factor known to regulate neurotrophin and neurotransmitter genes such as BDNF and somatostatin) mediates a cGMP/PKG-dependent anti-apoptotic signal cascade activated by NO (Nagai-Kusuhara et al., 2007; Zhuravliova et al., 2007; Riccio et al., 2006). As discussed previously ADNP and VIP were found to have neuroprotective

properties and we have recently shown ADNP epxpression in neurons, (in the cytoplasm and axons) and in glial cells of the rat brain (Cosgrave et al., 2008; Gennet et al., 2008). These proteins may represent new targets for brain injury and a potential mechanism of action via which these proteins and their gene expression may be regulated may be via the NO-cGMP pathway. Therefore, using the general NOS inhibitor L-NAME, the nNOS inhibitor 7-NI and the sGC inhibitor ODQ in this thesis I have investigated the regulation of ADNP and VIP expression via the NO-cGMP pathway following seizure.

1.13 Aims of Thesis

As mentioned previously, one of the main changes observed in the hippocampus associated with seizures includes the altered expression and release of neurotransmitters/ glial trophic factors leading to neurological changes in the brain. The overall aim of this project was to investigate the NO-cGMP pathway in the regulation of gene and protein expression of ADNP and VIP at basal levels and following KA-induced seizure. Hence:

- ADNP expression and regulation via the NO-cGMP pathway was studied in the hippocampus and DG following seizure.
- VIP expression and regulation via the NO-cGMP pathway was studied in the hippocampus and DG following seizure.
- As previously discussed, the DG is one of the few sites of postnatal neurogenesis in the brain and so the role of NO and ADNP in neurogenesis and cell proliferation/neurotrophic effects was also investigated.

CHAPTER 2

Materials and Methods

2.1 Animals

Experiments were carried out on 30-40 day old male Wistar rats (Biomedical Services Unit, University of Liverpool, UK) preferably from the same litter. Rats were housed under controlled environmental conditions (19-23 ^{o}C, 12 hrs light, 12 hrs dark) with food and water available *ad libitum*. Animals were culled according to local and national Schedule I requirements and all procedures carried out in compliance with the Animal Scientific Procedures Act (1986).

2.1.1 In Vivo Nitric Oxide Synthase Inhibitor and Kainic Acid Experiments

A circadian variation in nitric oxide synthase (NOS) response to NOS inhibitors has previously been reported, in that NO levels are higher during the day than at night (Dzoljic et al., 1997). Also NO production displays a cyclic pattern in the hippocampus (Bush & Pollack, 2001). It has previously been shown that multiple-dose administration of NOS inhibitors can result in inhibition for an extended period of time (Bush & Pollack, 2001), hence in order to achieve maximum NOS inhibition a 3 dose regimen was carried out 24 hrs prior to KA (Tocris,UK) treatment (10 mg/kg i.p.) where L-NAME (Tocris, UK) (50 mg/kg i.p.), 7-NI (Sigma,UK) (50 mg/kg i.p.) and ODQ (Tocris, UK) (10 mg/kg i.p.) were administered 3 times over the duration of the 24 hrs (Fig. 2.1). Drug concentrations were determined from the literature (Borowicz et al., 2000; Di Matteo et al., 2006; Kawaguchi et al., 1999; Martínez-Quiroz et al., 2005). Time points were also derived from the literature, mRNA and cellular changes were observed by 3 hrs and 3 days, respectively (Mikuni et al., 2001; Faherty et al., 1997; Najm et al., 1998; Lee et al., 1997). Following KA treatment, seizures were classified according to the Racine scale (Racine, 1972) and upon the manifestation of a full generalised seizure, half of the animals were euthanized (no more than 3 hrs post KA). The other half were administered diazepam (10 mg/kg i.p.) after the initial seizure to avoid SE or mortality. These animals were allowed to recover and 3 days later tissue was collected. For each time point 3 hrs and 3 days half the animals from each group were perfused intracardially with 4% paraformaldehyde (PFA), the brains dissected and post-fixed in 25% sucrose for immunostaining. The other half of the group were euthanised by schedule 1 and the

hippocampi and/or the dentate gyri were taken for RNA extraction (one hippocampus) and protein analysis (the other hippocampus).

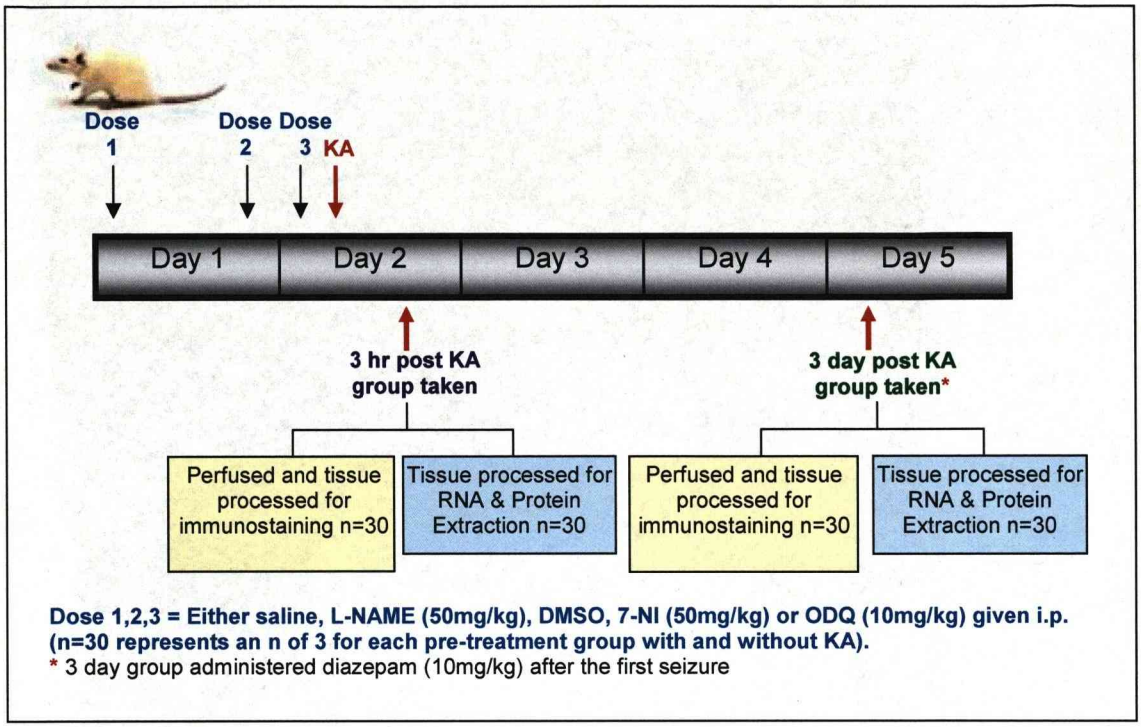


Figure 2.1 Schematic representation of the protocol carried out for in vivo experiments. A total of 120 animals were used and injected with either; vehicle control (saline/10% DMSO), L-NAME (50 mg/kg), 7-NI (50 mg/kg) or ODQ (10 mg/kg) (n=24). Half of the animals from each pre-treatment group were then given kainic acid (KA; 10 mg/kg) and taken at 3 hrs or 3 days post KA (along with those not given KA i.e. pre-treatment controls). For both time points tissues were processed for immunostaining or RNA and protein extraction. n=30 represents an n of 3 for the 5 pre-treatment groups with and without KA. See Table 2.1.

Saline (vehicle control for L-NAME) n=3	**Saline +KA** n=3	**L-NAME** n=3	**L-NAME +KA** n=3	**DMSO** (vehicle control for 7-NI and ODQ) n=3	**DMSO +KA** n=3	**7-NI** n=3	**7-NI +KA** n=3	**ODQ** n=3	**ODQ +KA** n=3

Table 2.1 n numbers for each pre-treatment with and without kainic acid (KA)

2.2 Nitrite Detection

2.2 Nitrite Detection

The Greiss assay is a popular assay for the detection of NO in tissue samples and biological fluids. It specifically measures nitrite (NO_2-), one of two primary, stable and nonvolatile breakdown products of NO. It is based on the diazotization reaction originally described by Greiss in 1897. The Greiss Reagent System (Promega, UK) uses sulfanilamide and *N*-1-napthylethylenediamine dihydrochloride (NED) under acidic (phosphoric acid) conditions. The sulfanilamide solution and NED solution were allowed to equilibrate at RT for 15-30 minutes. A Nitrite Standard reference curve was produced by diluting the 0.1M Nitrite Standard 1:1000 to give 100 µM nitrite solution, 100 µl of this was pippetted in triplicate into the first row (A) of a 96 well plate. 50 µl of the appropriate buffer (that samples were collected in) was added in triplicate to rows B-H. 2-fold serial dilutions were then immediately performed by pipetting 50 µl from row A to row B, mixing then pipetting 50 µl from row B to C and so on until row G where 50 µl was discarded from each well in this row and row H contained no nitrite solution to give a blank of 0 µM. Ultimately the nitrite concentration range was 0-100 µM. 50 µl of each experimental sample was then added to the remaining wells in triplicate. Using a multichannel pipettor and taking care not to contaminate samples, 50 µl of the sulfanilamide solution was then added to all wells containing standards and samples and incubated for 5-10 minutes at RT, protected from light. 50 µl of the NED solution was then added and incubated for 5-10 minutes at RT, protected from light. Within 30 minutes a purple coloured azo compound was formed and the optical density was measured at 540 nm on a scanning multiwell spectrophotometer (Thermofisher Scientific, UK). The nitrite concentration was quantified in Excel (Microsoft) by interpolating the standard curve as previously described (BCA assay).

2.3 Tissue Processing for Immunofluoresence

2.3.1 Cryopreservation

Animals were euthanized using halothane and perfused intracardially with 4% PFA in 0.1M phosphate buffered saline (PBS) pH 7.4.) 0.1M PBS was prepared from a 0.2M stock of sodium dihydrogen phosphate ($NaH_2PO_4.H_2O$) and a 0.2M stock of disodium

hydrogen orthophosphate ($Na_2HPO_4.12H_2O$) with 0.15M NaCl in distilled water (DW). Brains were then dissected out and further post-fixed in 4% buffered PFA for 4 hrs at 4°C, cryo-preserved in 25% sucrose for a few days and then gelatin embedded.

2.3.2 Gelatin Embedding of Tissue

Gelatin solution containing 7.5% porcine gelatin (Sigma, UK), 15% sucrose (BDH, UK) and 0.1% sodium azide (BDH, UK) in PBS was heated to 45-50°C. This was then cooled to 38°C and tissues incubated in a labeled petridish containing gelatin for 3 hrs at 38°C. After this time the tissue was left overnight at 4°C to allow the gelatin to set.

2.3.3 Chrome Alum Gelatin (CAG) Coating of Slides

A gelatin solution containing 0.5% porcine gelatin (Sigma, UK), in ddH_20 was heated to 45-50°C and 0.8% chrome alum (Sigma, UK) dissolved in the solution. 0.25% sodium azide (BDH, UK) was then added and the solution filtered and stored at RT. Slides were dipped in the CAG solution and left to air dry in dust free conditions overnight in a class B fume hood. Once dry, slides were stored in boxes at RT.

2.3.4 Cryosectioning

Gelatin embedded tissues were blocked and stuck onto a labelled cork disk using TissueTek® (Alpha labs, UK) and frozen in liquid nitrogen cooled isopentane prior to sectioning on a cryostat. The brains were cut into 15 µm sections and mounted onto chrom alum coated slides (BDH Laboratory Supplies, UK). Sections were collected consecutively from the start of the hippocampus onto 10 different slides and then collected again onto the first slide. Four sections per slide were collected so that on any given slide there was a 600 µm range throughout the hippocampus. As shown in Figure 2.2. The brain sections were allowed to dry onto the slides at RT for 30 minutes, then stored at -40°C.

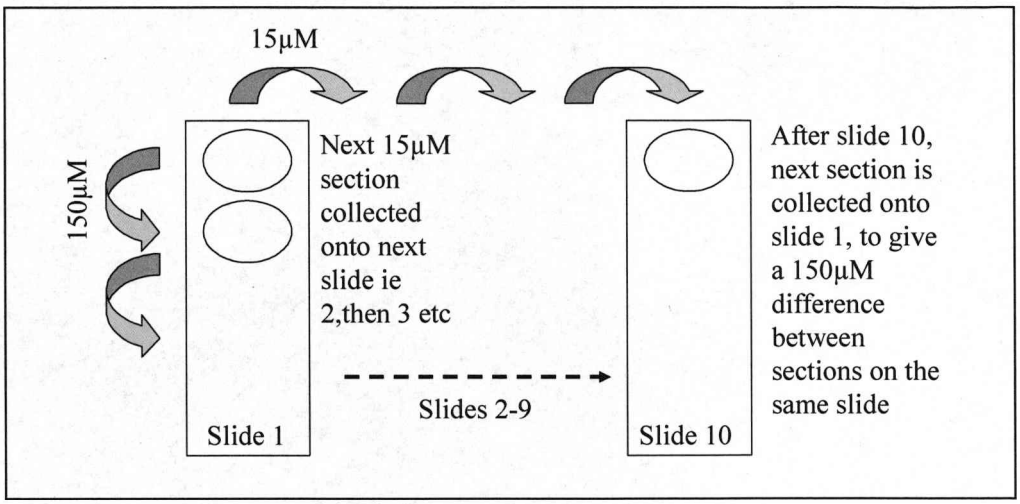


Figure 2.2 Schematic representation to show the chronological order of sections on each slide, so that each slide contains an approximate cross-section of 600 μm throughout the brain.

2.3.5 Immunofluorescence

Sections or dissociated hippocampal cultures were washed in PBS for 10 minutes (x3) at RT to remove PFA. Sections/cultures were then blocked with 10% donkey serum (DS) for 1hr at RT. Double or triple staining was carried out sequentially, all antibodies were diluted in diluting solution (0.1M PBS, pH 7.2, 0.1% Triton X-100, 2% DS and 0.1% sodium azide), 3-4 washes with PBS were applied between each step. For immunostaining, the appropriate concentrations of primary antibodies were applied overnight at 4°C (Table 2.2). The next day donkey raised appropriate secondary antibodies either biotinylated or flourochrome-conjugated (Jackson Immunoresearch Laboratories, Inc) were applied at for 1hr at RT, washed 3x in PBS, followed by the appropriate streptavidin conjugated-FITC/Cy3/Marina blue (from Vector Laboratories, Jackson Immunoresearch Laboratories, Inc and Molecular Probes, respectively) for 1hr at RT and then washed 3x in PBS (Table 2.3). For triple immunostaining, a third primary antibody was applied overnight at 4°C and processed as previously described with the appropriate secondary antibody. Sections/cultures were then covered with VectaShield (Vector Laboratories), coverslipped and then viewed using a Nikon inverted microscope (Nikon,UK). Images were captured using IPL lab software (Nikon,UK). Sections were

(Nikon,UK). Images were captured using IPL lab software (Nikon,UK). Sections were covered with VectaShield (Vector Laboratories), coverslipped and viewed with a Nikon inverted microscope (Nikon,UK) using the appropriate wavelength filter for each fluorochrome and images merged using IPL lab software (Nikon, UK).

Antibody	Species Raised In	Primary Antibody Dilution	Source
Anti-nNOS	Sheep	1:1000	Dr P.C. Emson
Anti-iNOS	Rabbit	1:300	Chemicon
Anti-ADNP	Rabbit	1:300	Chemicon
Anti-VIP	Rabbit	1:200	Chemicon
Anti-GFAP	Rabbit/ Mouse	1:200	Sigma
Anti-NeuN	Mouse	1:50	Chemicon
Anti-BrdU	Mouse	1:100	Molecular Probes

Table 2.2 Details of primary antibodies used for immunostaining.

Antibody	Species Raised In	Secondary Antibody Dilution	Source
Anti-Mouse Biotinylated	Donkey	1:200	Jackson ImmunoResearch
Anti-Rabbit Biotinylated	Donkey	1:500	Jackson ImmunoResearch
Anti-Sheep Biotinylated	Donkey	1:200	Jackson ImmunoResearch
CY3 Anti-Rabbit	Donkey	1:200	Jackson ImmunoResearch
Streptavidin CY3	-	1:200	Jackson ImmunoResearch
Streptavidin FITC	-	1:80	Vector Laboratories
Streptavidin Marina Blue	-	1:100	Molecular Probes

Table 2.3 Details of secondary antibodies used for immunostaining.

2.6 TUNEL Staining

For the detection of apoptosis at the single cell level, Terminal Transferase (TdT) and Biotin-16-dUTP (TUNEL) staining is often employed. This method labels double stranded and single stranded DNA breaks (nicks) which occur during apoptosis. The free

3'-OH terminal is labelled with modified nucleotides in an enzymatic reaction. For positive controls sections were incubated with DNase I (3000 U/ml in 50mM Tris-HCl, pH 7.5, 1 mg/ml BSA) for 10 mins at RT to induce DNA strand breaks prior to the labeling procedure. Also sections from the rat intestine ileum were used as known positive controls. For negative controls sections were incubated with label solution only instead of TUNEL reaction mixture. Sections were washed 3 times for 10 mins in ddH_2O then incubated with 0.2% Triton X-100 in PBS-Tween for 30 mins. Sections were then treated with proteinase K (10 µg/ml) at RT for 15 mins and then washed twice in PBS-Tween for 2 mins. Then, sections were incubated in TdT Reaction Buffer (25mM Tris-HCl, 200mM, Sodium Cacodylate, 0.25 mg/ml BSA, 1mM Cobalt Chloride) for 10 mins and then incubated in TdT Reaction Mixture which was composed of 100 µl solution A (TdT (Roche Diagnostic, UK) 4 µl in 100 µl TdT Storage buffer (60mM potassium phosphate, pH 7.2, 150mM KCl, 1mM 2-Mercaptoethanol, 0.5% Triton X-100, 50% glycerol) and 100 µl solution B (4 µl Biotin-16-dUTP (Roche Diagnostic, UK) in 1ml TdT Reaction Buffer) for 2 hrs at 37°C in a humidified chamber. Sections were then incubated in Stop Wash Buffer (300 mM NaCl, 30 mM Sodium Citrate) for 10 mins and then washed 3 times in PBS-Tween for 2 mins. Immunofluorescence was then carried out by incubating the sections in streptavidin-FITC (Vector Laboratories) at 1:80 for 1 hr at RT and then washed 3 times in PBS-Tween and mounted with Vectashield.

2.7 Cell Quantification

Three concurrent sections per rat from each of the cranial, middle and caudal $1/3^{rd}$ region of the hippocampus were selected for counting from control and drug treated samples that were processed for double/triple immunostaining simultaneously using the same reagents and/or antibodies. All slides were given a code and the code revealed after statistical analysis for unbiased cell counting. The appropriate wavelength filter for each fluorochrome was used to visualize immunopositive cells, the image captured and then the filter was changed (without changing the field of view) to view a second or a third marker. Using IPL image analysis software, a threshold was set for each fluorochrome and the same threshold was used for both drug and appropriate control. A minimum of three animals from each group was used for cell counting. The average cell numbers were

calculated for each animal, the SEM calculated for each treatment group and p values obtained using one-way ANOVA with Bonferroni post-hoc test a 'p' value of less than 0.05 was considered to be significant. (*p <0.05, **p <0.01, ***p <0.001). (Graphpad software Inc, USA)

2.8 Tissue Culture Methods

2.8.1 General Cell Handling

All cell culture work was carried out under aseptic conditions in a class II laminar flow cabinet. Cells were cultured in 24 well plates (Greiner bio-one, UK) and incubated at 37°C in water-saturated air with 5% CO_2. All media was filter sterilised through a 0.22 μm filter (Millipore, USA) and pre-warmed to 37°C in an incubator (Sanyo, Japan) prior to plating or media changes.

2.8.2 Preparation of 24- well plates

Poly-D-Lysine solution, 300 μl (0.1 mg/ml; Sigma, UK) was added to each well of a 24 well plate and incubated in the laminar flow hood at RT for 1 hr. The poly-D-lysine was then removed and the wells washed three times with 500 μl of sterile distilled water (DW), each wash was for 20 minutes. After the third wash the Dw was removed and the plates left to completely dry in the laminar flow cabinet for at least 1 hr.

2.8.3 Dissection of Dentate Gyri

All surgical instruments were cleaned and sterilised by autoclaving prior to dissection. All dissections were carried out in a horizontal laminar flow cabinet. Experiments were carried out on 6-9 day old male Wistar rats (Biomedical Services Unit, University of Liverpool) preferably from the same litter. Following culling of the animals in accordance with Schedule I of the Animal Scientific Procedures Act (1986), the brain was exposed and carefully removed into a petridish containing chilled dissection media, Geys balanced salt solution (Sigma, UK) containing 4.5 mM glucose (Sigma,UK) (Appleton Woods, UK). The cerebellum and brain stem were removed and the brain split sagitally into the two hemispheres to expose the hippocampi. The hippocampus from each hemisphere was then removed using paddle pastettes (Alpha labs, UK) and placed

onto a melinex strip (Agar Scientific, UK), a polyester film approximately 5 cm by 4 cm. The aligned hippocampi were sliced using a McIlwain tissue chopper (Mickle Laboratory Engineering Co. Ltd., UK) to give 1 mm thick transverse slices. The slices were then placed into fresh dissection media and the dentate gyrus was cut from the rest of the hippocampus with a sterile needle (Terumo, UK) and blade (Appleton woods, UK) (Fig. 2.3) under a dissecting microscope (Zeiss, UK). The dentate gyri and hippocampi were collected on ice into separate 6 cm petridishes containing dissection media.

2.8.4 Dissociation and Plating

The dissection medium was carefully removed from the dishes and tissues were washed in approximately 3-5 mls of Neurobasal-A supplemented with 2% B27+ media (NB/B27+). This was then removed and replaced with NB/B27+ containing papain (2 mg/ml) (Sigma, UK) to aid enzymatic digestion and cell release and incubated for 30 min at 37°C, agitating every 10 mins. After 30 mins the NB/B27+ containing papain was carefully removed, and cells washed twice with NB/B27+. The cells were then triturated by pipetting approximately 10 times until a homogenous cell suspension was acquired and then transferred to a 15ml centrifuge tube. An OptiPrep gradient was prepared by gently pipetting 10% Optiprep (made up in NB/B27+) on top of 20% Optiprep. The cell suspension was then applied gently to the top of this 2-step density OptiPrep gradient and centrifuged for 15 mins at room temperature at 1900 rpm to partially separate cells from debris. The fraction between the 10% and 20% gradient containing the cells was gently pipetted out and diluted into 2 mls NB/B27+ and centrifuged for 2 mins at RT at 1100 rpm. The supernatant was removed and the cell pellet was re-suspended in 4 mls NB/B27+. Following trituration, 30µl of cell suspension was added to 50 µl of trypan blue stain (Sigma, UK), mixed well, and counted using a haemacytometer. Viable cells were counted by trypan blue exclusion and diluted to have a cell density of 100,000 cells/ml, subsequently 500 µl was added to each well of a 24 well plate, previously coated with poly-D-lysine. A media change was carried out the next day to remove any cell debris and non-adherent cells. Plating media contained a combined antibiotic/antimycotic 250 µM (Pen/Strep and Fungizone, Gibco, UK), 500 µM L-Glutamine (Sigma, UK) and 10% FCS (Gibco, UK). After three days FCS was excluded from the media to promote

neuronal differentiation, and thereafter a media change was carried out every 3 days. Immunofluorescent staining on cultures was carried out as previously described.

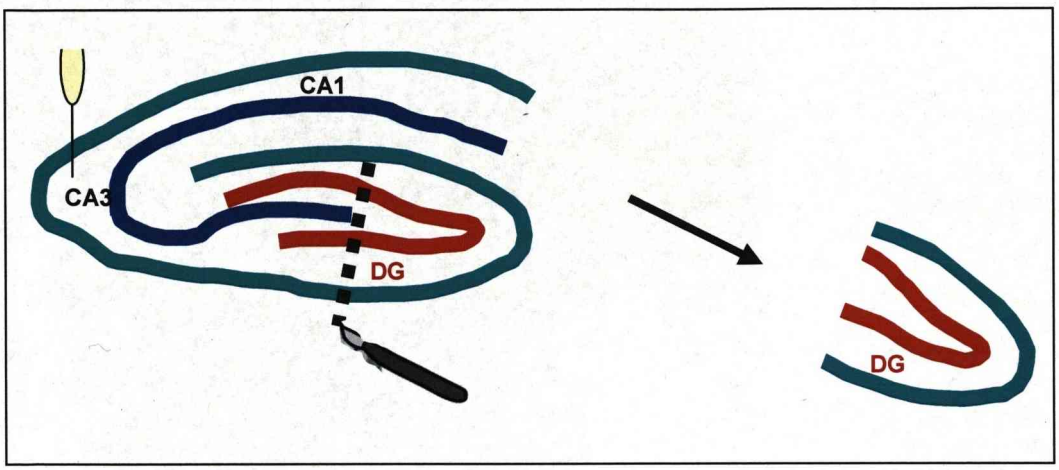


Figure 2.3 Schematic diagram illustrating the removal of the dentate gyrus from the hippocampus. A needle was placed in the CA3 region to hold down the section whilst using a scalpel to cut and lever out the dentate gyrus from the rest of the hippocampus.

2.9 Molecular Biology Methods

2.9.1 General Molecular Biology Procedures

All plasticware, glassware, equipment and working areas were autoclaved or cleaned with RNase Away (Molecular Bioproducts, UK) and PCR was carried out in a PCR laminar flow cabinet (Labcaire Systems Limted, UK) to reduce contamination of samples.

2.9.2 RNA Extraction

Experimental and control tissues were collected in 1ml of Trizol (Invitrogen, UK) /50 mg tissue, homogenised as rapidly as possible using decreasing sized gauge needles on ice at 4°C, until a smooth, lysed homogenous suspension was obtained. For cells, the media was removed and cells washed with chilled PBS which was then removed and 250 µl of trizol added to each well (for a 24-well plate). The cells were then scraped using a pipette tip and placed in a 1.5 ml eppendorf (Appleton Woods, UK). In a fume hood, 200 µl of chloroform per 1 ml of Trizol was then added. The suspension was shaken vigorously for 15 seconds and incubated for 3 minutes at RT. The suspension was then spun down at

12,000 g for 15 minutes at 4°C and the supernatant removed and transferred to a sterile (RNase free) eppendorf. DNA and protein were removed through salt-precipitation, by adding 500 µl of isopropanol per 1 ml of Trizol. The RNA was allowed to precipitate at RT for 15 minutes and then spun down at 12,000 g for 15 minutes at RT. The supernatant was discarded and the RNA pellet washed twice with 1ml of 75% ethanol. The pellet was vortexed after each wash and spun at 7,500 g for 3 minutes at 4 °C to remove any residual ethanol. The RNA pellet was allowed to dry at RT for 10 minutes and resuspended in RNase free water 1 µl per 1mg tissue. This was then vortexed, left on ice for 10 minutes, heated to 65°C for 10 minutes and left on ice before storage at -80°C.

2.9.3 DNase Digestion

RNA was digested with RQ1 RNase-free DNase (Promega, UK), to remove DNA contaminants. 8µl of RNA was digested with 1µl (=1unit) RQ1 RNase-free DNase and 1µl of RQ1 10x buffer (Promega, UK), in a total volume of 10µl at 37°C for 30 minutes. 1µl of Stop-solution (Promega, UK) was then added and the enzyme inactivated at 65°C for 10 minutes. Successful removal of DNA was determined by visualisation of RNA and DNase treated RNA on a 1% agarose gel via electrophoresis. The concentration of each sample of RNA was determined with a Gene Meter spectrophotometer (ABgene, UK). A 1:100 dilution of the RNA to water was used to determine the concentration and water alone was used as a no RT-control for semi-quantitative PCR analysis (Absorbance A260/280 x dilution factor x 40). 200 ng of DNase digested RNA was used for reverse transcription.

2.9.4 Reverse Transcription

Reverse Transcription using Invitrogen's SuperscriptII system was carried out on 200 ng of RNA for each sample and the manufacturer's instructions followed. Initially, RNA was mixed with dNTP's and oligo dT as follows:

Oligo (dT) 12-18 (500 ug/ml)	1 µl
dNTP mix (10 mM)	1 µl
RNA/control	variable
RNase-free water	variable
Total volume:	12 µl

The reaction was heated to 65°C for 5 minutes, chilled on ice immediately and spun down. Then to each sample added:

5x First Strand Buffer	4 µl
DTT (100mM)	2 µl
RNaseOUT Recombinant Ribonuclease Inhibitor (40units/ul)	1 µl

Each tube was inverted and reaction mixed gently, this was then incubated at 42 °C for 2 minutes, 1 µl of SuperscriptII (200 units) was then added to each sample and incubated at 42 °C for a further 50 minutes. The reaction was then inactivated at 70 °C for 15 minutes and kept at 4 °C for 5 minutes to keep the cDNA in a denatured state. For quantitative PCR (qPCR), the cDNA concentration was determined with a spectrophotometer (Absorbance A260/280 x dilution factor x 50) and diluted to 25 ng/µl stocks in nuclease-free water. The cDNA was stored at -20° C and subsequently 8 µl (200 ng) was used as a template for qPCR.

2.9.5 Semi-Quantitative Polymerase Chain Reaction (PCR)

Polymerase Chain Reaction (PCR) was used for the semi-quantitative analysis of mRNA expression. PCR was performed in an Eppendorf Mastercycler Gradient (Eppendorf,UK) thermal cycler. Taq DNA polymerase was obtained from Promega, dNTPs were from Invitrogen and prepared as a stock solution containing 10 mM of each dNTP. Primers were obtained from MWG Biotech, Germany and reconstituted with the appropriate volume of RNase free H_2O stated in the oligo synthesis report to give a 100 µM stock and stored at -20°C. All primer stocks were then diluted to 10 µM concentration stocks for direct use in PCR and qPCR. Two microlitres of cDNA was used as template in a standard 50 µl PCR reaction with Taq DNA polymerase as follows:

Template	2 µl
10x PCR buffer	5 µl
$MgCl_2$ (50mM)	1.5 µl
dNTP mix (10mM)	1 µl
Forward primer (100µM)	1 µl
Reverse primer (100µM)	1 µl
Taq DNA polymerase	0.4 µl
ddH_2O	38.1 µl
Total volume:	50 µl

Reaction mixtures were prepared in 0.2ml capped thin walled tubes (Eppendorf, UK). PCR programmes were adapted from a standard protocol. Rat ADNP and VIP mRNA levels were carried out with ADNP forward 5'-GGA CCA CAT TGT CAA TTC ACA CC-3' and ADNP reverse primer 5'-GGA CAA GCG CTG CAG CAG AAA GG-3', VIP forward primer 5'-TAA AAG CAG ACT CTG ACA TCT TG-3' and VIP reverse primer 5'-GAA GTT GTT TTC TTG AAT TAG AT-3' with 40 amplification cycles. mRNA levels for nNOS and iNOS were carried out with nNOS forward primer 5'-GAA CCC CCA AGA CCA TCC and nNOS reverse primer 5'-GGT TTG CTC CCA CTG TT-3'. iNOS forward primer 5'-CAC ATC TGG CAG GAT GAG AA-3' and iNOS reverse primer 5'-TTG TCA CCA CCA GCA GTA GTT-3'. As a control for the amount of cDNA used in the reactions, rat β-actin levels were also analysed, using b-actin forward 5'-ACG GTC AGG TCA TCA CTA TGG-3'and reverse primers 5'-AGC CAC CAA TCC ACA CAG-3'. Most PCR programmes were as follows:

Temperature	Time	Number of Cycles	Function
94°C	5 minutes	1	Initial denaturation
94°C	1 minute	40	Denaturation
60°C	1 minute		Annealing
72°C	1 minute		Extension
72°C	5 minutes	1	Strand completion

Table 2.4 Details of PCR programmes.

2.9.6 Agarose Gel Electrophoresis

PCR products were visualised by agarose gel electrophoresis. Agarose (1%) (Bioline,UK) was melted in 0.5x TBE buffer in a 250 ml Erlenmeyer flask, by boiling for 20 seconds in a microwave and then cooled to approximately 55°C. 4μl of ethidium bromide was then added (10mg/ml aqueous solution) (Sigma, UK) to the gel. Gels of 150ml were cast in 12x14cm or 20.5x10cm trays, which were taped either end, and appropriate combs inserted. The gel was allowed to set for approximately 30 minutes at RT and was then submerged in a horizontal gel electrophoresis tank (Hybaid turn and cast submarine gel system, Hybaid, or Savant HG 350 tank) containing 0.5x TBE buffer and the combs carefully removed. Samples were mixed with 6x loading buffer (AB gene, UK) and loaded into the wells. Five microlitres of 100bp DNA ladder (AB gene, UK) was also

loaded to compare the size of products. The gel was then run at 120-150V (Hybaid) or 80V (Savant).

2.9.7 Generation of Standards for Quantitative Real Time PCR (qPCR)

Plasmid standards provide a method for determining gene copy numbers in qPCR. All standards for qPCR were generated and kindly donated by Dr Nicole Gennet. The standards were generated as follows; β-actin and ADNP cDNA PCR products were produced as described in (Reverse Transcription section) and (PCR section). The PCR products were ran on a 1% agarose gel and excised from the gel using a sterile scalpel blade under a UV transluminator. The DNA was then purified from the agarose using the QIAquick Gel Extraction Kit (Qiagen, UK) and cloned into the pGEM®-T vector system (Promega, UK). Different insert:vector ratios were used, ranging from 5:1 to 1:5. The amount of insert required was calculated using the following calculation:

$$\frac{\text{ng vector} \times \text{Kb size of insert}}{\text{Kb size of vector}} \times \frac{\text{insert ratio}}{\text{vector ratio}} = \text{ng of insert}$$

The appropriate volume of insert and 1 µl (50ng) of pGEM®-T vector DNA were mixed together with 1 µl 10x ligase buffer and 1 µl of T4 DNA ligase (3 units) (Promega, UK) in a total volume of 10 µl and incubated at 4°C overnight in a 1.5 ml centrifuge tube. Transformation of the plasmid DNA into *Escherichia coli* JM109 (Promega, UK) cells was as follows; cells were thawed on ice and 2-3 µl of the ligation reaction, added. This mixture was incubated on ice for a further 30 min, then heatshocked at 42 °C for 45 seconds in a waterbath and incubated on ice for 2 min. 950 µl of warmed Luria-Bertani Broth (LB) medium was added to the cells and the culture incubated at 37 °C for 1 hour on a shaker at 225 rpm. 100 µl of this culture was then spread onto LB agar plates (containing 100 µg /ml ampicillin) which had previously been streaked with 40µl of X-gal solution (50mg/ml, Promega) and 16µl of IPTG (Promega, 100mM) per 10cm and incubated at 37°C overnight. The next day, white positive colonies were picked and analysed for the correct insert. A small scale preparation of plasmid DNA for each colony was carried out using the QIAprep Spin Miniprep Kit (Qiagen, UK). A restriction endonuclease digest was then carried out to establish the correct ligation and a large scale

preparation of the correct plasmid was then performed using the QIAGEN Plasmid Maxi Kit (Qiagen, UK).

The plasmid DNA concentration was determined by reading absorbance at A_{260} using a spectrophotometer. By knowing the concentration and the size of the overall construct, the copy number could then be determined using the following equation:

$$\text{Copy No per } \mu l = \frac{[\text{grams of DNA}] \times [6.022 \times 10^{23}]}{\text{construct size (bp)} \times 660^*}$$

*660 = M_r of a nucleotide.

Serial dilutions of the plasmids ranging from 1.0×10^8 to 1.0×10^3 copies were produced for ADNP and the β-actin housekeeping gene.

2.9.8 Quantitative Real Time PCR

Stocks of standards were thawed on ice along with cDNA samples, reactions were set up in a pre-cooled 96 well ice block. 1 µl of each standard for ADNP or β-actin was mixed with 7 µl H_2O. 12µl of master mix (1µl H_20, 0.5µl forward primer, 0.5µl reverse primer and 10µl Finnzymes DyNAmo™ SYBR® Green master mix (containing modified *Thermus brockianus* (Tbr) DNA polymerase, SYBR Green I, optimized PCR buffer, 5mM $MgCl_2$, dNTP mix including dUTP)) was added to each standard and to 200ng (8µl) of each cDNA sample to give a total volume of 20 µl.

qPCR cycling steps were carried out as follows; block pre heating 94°C for 10 minutes, then 45 cycles of; initial denaturation 94°C for 10 minutes, annealing 60°C for 20 seconds, extension at 72°C for 15 seconds and strand completion 72°C for 1 second. Continous fluorescence was detected using the DNA Engine Opticon™ System (CFD 3200, Opticon detector and PTC 200 DNA engine cycler) (MJ Research Inc.). SYBR Green I binds to double-stranded DNA and fluoresces when bound to the amplified double-stranded PCR product, allowing the direct quantification of amplified DNA (amplicons) without the use of labelled probes. A standard curve for each housekeeping gene or gene of interest was generated and used to calculate the relative copy number of unknown samples. Melting curve analysis was carried out to confirm the specificity of

the products between 65-95 °C with 0.2 °C increments. The content of unknown samples was calculated from the amount of the target gene, normalised to the amount of a housekeeping gene, β actin, with each derived from separate standard curves.

2.10 Protein Extraction

In vivo tissue samples were collected in 500 µl RIPA buffer (50 mM Tris-HCl pH7.4, 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 1% Protease Inhibitor Cocktail (all from Sigma, UK)) on ice. In vitro, media was removed and cells were washed with ice cold PBS, 200 µl of RIPA buffer was then added to the wells. Subsequently, the cells were scraped with a pipette tip and collected into a 1.5 ml eppendorf tube. Samples were then pipetted several times to aid lysis and the protein concentration then determined using the BCA assay.

2.10.1 Determining the Protein Concentration of Samples

To determine the protein concentration of samples for western blotting a protein assay was carried out using the bicinchoninic acid (BCA)^{TM} Protein Assay Kit (Pierce, UK). This assay involves the reduction of Cu^{2+} ions to Cu^{+} ions by protein under alkaline conditions, also known as the Biuret reaction. The Cu^{+} ions are then detected by a colorimetric reaction using BCA in which the chelation of two BCA molecules with one Cu^{+} ion generates a purple coloured reaction product. The intensity of the colour reaction can be used to determine unknown protein concentrations by comparing them to a set of standards with known protein concentrations. A set of standards with protein concentrations ranging from 25 µg/ml to 2000 µg/ml including a blank of 0 µg/ml were made up along with the working reagent (WR) which was made up from reagents A and B in a ratio of 8:1. The samples to be assayed were used neat, or diluted in order to achieve a concentration within the range of the standards. 25 µl of each standard and sample were pipetted in triplicate into a clear 96 well microplate and 200 µl of WR multi-pippetted into these wells, taking care to avoid contamination between wells. The plate was covered and incubated at 37°C for 30 minutes. The plate was then cooled to RT, and the optical density was measured at 540nm on a scanning multiwell spectrophotometer (Thermofisher Scientific, UK). The protein concentration was quantified in Excel

(Microsoft) by interpolating the standard curve using the linear equation $y = mx + c$ (where excel fits gradient and y intercept):

$$\text{Concentration} = \frac{[(\text{Absorbance})-(\text{y intercept})]}{\text{Gradient}}$$

2.10.2 Western Blotting

Proteins were resolved using the NuPAGE®Novex® 10% Bis-Tris pre-cast gels (Invitrogen, UK) and the XCell *SureLock*™ Mini-Cell electrophoresis system. The appropriate volume for 10 µg of each sample was added to an eppendorf then all samples were made up to an equivalent volume with ddH_2O and finally NuPAGE® LDS Sample Buffer (4X) and NuPAGE® Reducing Agent (10X) were added to the samples. Samples were then heated to 70°C for 10 mins. 1X SDS Running Buffer was prepared by diluting 20X NuPAGE® MOPS SDS Running Buffer in ddH_2O. The wells of the pre-cast gels were washed twice with 1X Running Buffer and then filled to displace air bubbles. The gel cassettes were then locked into the buffer chamber which were then filled with 1X running buffer. 35 µl of samples were loaded and 5 µl SeeBlue® Pre-Stained Standard ladder (Invitrogen, UK) and ran for 60 mins at 200 V (time varied according to the size of band being investigated). 1X NuPAGE® Transfer Buffer was then prepared by diluting 20X NuPAGE® Transfer Buffer in ddH_2O (800 ml) and adding 200 ml methanol and 1 ml NuPAGE® Antioxidant. The gels, nitrocellulose membranes (Invitrogen, UK), filter paper ((Invitrogen, UK) and blotting pads (Invitrogen, UK) were pre-soaked in 1X NuPAGE® Transfer Buffer and then locked into the blot module which was filled with 1X NuPAGE® Transfer Buffer, the outer chamber was filled with ddH_2O. Proteins were then electrophoretically transferred to the nitrocellulose membranes at 60V for 1 hr.

2.10.3 Processing of Western Blots

Detection of proteins was carried out using the WesternBreeze® Chromogenic Immunodetection Kit (Invitrogen, UK). Blocking solution was made up according to manufacturers guidelines using Diluent A and Diluent B and the nitrocellulose membranes blocked for 1 hr on a rotary shaker set at 1 revolution/ second. Membranes were then washed twice with dH_2O and incubated with primary antibodies (ADNP, 1:1000, Chemicon, UK; or β-actin 1:500, Sigma, UK) overnight at 4°C. The next day the

membranes were washed 3 times with Antibody Wash, prepared according to manufacturers guidelines, and then incubated with the Secondary Antibody Solution for 30 mins at RT. The membranes were then washed 4 times for 5 mins with Antibody Wash, then 3 times for 2 mins with dH_2O. The Chromogenic Substrate was then added to the membranes until purple bands developed (between 10- 30 mins), then the membranes were washed 3 times for 2 mins with dH_2O and left to air dry.

2.11 BrdU labeling

2.11.1Detection of Proliferating Cells In Vivo

5-bromo-2-deoxyuridine (BrdU) (100 mg/kg) was administered 3 days prior to KA and other pre-treatments carried out as described previously i.e. 24 hrs prior to KA treatment (10 mg/kg), L-NAME (50 mg/kg) and saline controls were administered 3 times over the duration of the 24 hrs (Fig 2.1). Following KA treatment, seizures were classified according to the Racine scale (Racine, 1972) and rats administered diazepam (10 mg/kg) after the initial seizure to avoid SE or mortality. These animals were allowed to recover and 3 days later the animals were perfused with 4% paraformaldehyde, the brains dissected and post-fixed in 30% sucrose for immunohistochemistry.

2.11.2 Detection of Proliferating Cells In Vitro

Dentate gyrus cells were cultured and maintained as described previously. To study the longer term effects of NO inhibition and KA treatment on proliferation *in vitro*, BrdU (10 μM) was added to the media along with either saline (vehicle control) or L-NAME (500 μM) and incubated for 30 mins at 37°C, 5% CO_2. KA (10 μM) was then added to either saline (vehicle control) or L-NAME (500 μM) treated cells and incubated for 3 days. Then the cells were fixed in 4% PFA and processed for BrdU immunostaining. For short term effects on cell proliferation cells were fixed 6 hrs post KA treatment.

2.11.3 BrdU Immunostaining

BrdU a thymidine analogue is incorporated into the newly synthesized DNA of proliferating cells both in vitro and in vivo and consequently antibodies specific to BrdU can be used to detect proliferating cells. Identification of the labelled cells can be applied

both in vitro and in vivo to produce a nuclear staining pattern. Tissue or cells were fixed with 4% PFA, in vivo tissue had previously been fixed with 4% PFA, for in vitro cultures the media was removed and cells were washed with 0.1M PBS, then 200 µl of 4% PFA was added to the cells for 30 mins at RT. Following fixation, cells/tissue sections were washed twice with PBS. For denaturing of DNA, the cells/tissue sections were incubated with 50% formamide in 2x SCC (0.3 M NaCl and 0.03m sodium citrate) at 65°C for 90 mins. After 90 mins the cells/tissue sections were washed twice with 2x SCC for 5 mins and then incubated for 30 mins in 2N HCl at 37°C. After 30 mins the acid was neutralized by washing the slides in 0.1M boric acid (ph8.5) for 10 minutes followed by five washes in PBS at RT. The cells/ tissue sections were then incubated with 10% donkey serum for 1 h at RT and the primary antibody anti-BrdU (mouse) at 1:100 incubated at 4°C overnight. Immunostaining was then carried out as described previously.

CHAPTER 3

The Regulation of ADNP via the NO-cGMP Pathway in the Hippocampus Following Seizure

3.1 Introduction

Activity dependent neuroprotective protein (ADNP) is widely expressed in many tissues, including heart, skeletal muscle, kidney, and placenta. It is also expressed in the brain in regions such as the cerebellum and cortex, where its expression is higher compared to other tissue (Zamostiano et al., 2001). The human ADNP gene is approximately 45 kb and contains 13 alternatively spliced exons which produce 5 splice variants (a-e). These have not been fully characterised, but four of these five splice variants are likely to produce the same protein, only splice variant (e) is significantly different in that it does not contain either of the alternative exons 10-13 which encode the largest portion of the ADNP protein. All ADNP references herein, refer to splice variant (c). Cloning of the ADNP gene also revealed that the deduced protein structure contained nine zinc fingers, a proline-rich region, a nuclear bipartite localization signal, and a homeobox domain profile, suggesting a transcription factor function of ADNP (Zamostiano et al., 2001). Part of the ADNP gene sequence was found to code for the eight amino acid peptide NAP (short for NAPVSIPQ, its one letter amino acid sequence) (Bassan et al., 1999; Zamostiano et al., 2001). NAP was then shown to have a potent neuroprotective function, even at femtomolar concentrations (Bassan et al., 1999; Zemlyak et al., 2000; Gozes et al., 2003; Furman et al., 2004; Zaltzman et al., 2005). In a mouse model of head trauma, NAP protected neurons from death (Gozes et al 2005; Zaltzman et al., 2005). In another study using the hydrogen peroxide-induced stress model, ADNP reduced the pro-apoptotic p53 protein in rat pheochromocytoma (PC12) cells, implying its role in programmed cell death (Steingart and Gozes, 2006). Importantly, a recent study by Vulih-Shultzman and colleaugues demonstrated that heterozygous $ADNP^{+/-}$ mice have increased phosphorylated tau, the hallmark of neurodegenerative diseases (Vulih-Shultzman et al., 2007). ADNP has also been shown to be essential for brain formation and development (Pinhasov et al., 2003; Mandel et al., 2007), however, its role in the pathophysiology of disease processes such as epilepsy has not been investigated.

The KA animal model of epilepsy, a model resembling human TLE, has been previously discussed. As described in the general introduction, analysis of hippocampal pathology in human TLE exhibits neuronal loss and gliosis (Nadler et al., 1978; Ben-Ari, 2000; De

Lanerolle et al. 2003). Gene expression in TLE samples is also consistent with increased glutamate release by astrocytes (Lee et al., 2007). The relationship of NO in epilepsy and hippocampal cell death is not completely understood as it has been shown to have both anti-convulsant (Penix et al., 1994, Kendrick et al., 1996; Gabriel et al., 2000; Sardo and Ferraro, 2007; Royes et al., 2007) and pro-convulsant actions (De Sarro et al., 1993; Tutka et al., 1996), depending on the species studied and epilepsy model employed. Several factors may contribute to hippocampal cell death in epilepsy such as excessive glutamate realease and activation of NMDA receptors leading to calcium-induced cell death. The calcium influx activates neuronal nitric oxide synthase (nNOS) to produce NO (Garthwaite, 2005). All three NOS isoforms are expressed during epilepsy, for example, eNOS is upregulated in a rodent model of SE within 3-24 hours of intracranial injection of KA (Chuang et al., 2007; Liu et al., 2007), whilst nNOS and iNOS are upregulated in a mouse model of electrically-induced SE (Catania et al., 2003). NO activates soluble guanylyl cyclase (sGC) to generate cGMP which can initiate physiological responses in the cell, primarily via the phosphorylation of proteins by protein kinases. NO can also directly modulate the expression of transcription factors, for example, CREB (a transcription factor known to regulate neurotrophin and neurotransmitter genes such as BDNF and somatostatin) mediates a cGMP/PKG-dependent anti-apoptotic signal cascade activated by NO (Nagai-Kusuhara et al., 2007; Zhuravliova et al., 2007; Riccio et al., 2006).

We have recently shown ADNP expression in neurons, (in the cytoplasm and axons) and in glial cells of the rat brain (Cosgrave et al., 2008; Gennet et al., 2008). In this chapter I have investigated the regulation of ADNP expression via the NO-cGMP pathway in the hippocampus, following seizure.

3.2 Materials and Methods

Materials and methods were carried out as previously described for in vivo injections of NO inhibitors and KA. Tissues were processed for immunofluorescence, PCR, qPCR and western blotting and measurement of nitrite also as described in the methods section.

3.3 Results

3.3.1 Effect of NOS or sGC inhibition on nitrite levels in the hippocampus and following the onset of the first seizure

Nitric oxide (NO) has a very short half life in vivo and is predominantly converted to nitrate (NO_3^-) and nitrite (NO_2^-). These products are more stable and so NO activity could be assessed by quantitating NO_2^- levels in hippocampal homogenates using the Griess assay. L-NAME (50mg/kg), 7-NI (50mg/kg) and ODQ (10mg/kg) pre-treatment all caused a significant decrease in NO_2^- levels compared to their vehicle control (Fig. 3.1). Following KA treatment and after the first seizure there was a significant increase in NO_2^- levels (Fig. 3.1) compared to vehicle control, this is consistent with previous findings (Radenovic & Selakovic 2005). Only L-NAME pre-treatment caused a significant decrease in NO_2^- levels following the first seizure, whereas 7-NI and ODQ pre-treatment did not have any significant effect on NO_2^- levels following the first seizure compared to vehicle control (Fig. 3.1).

3.3.2 Kainic acid treatment increases nNOS and iNOS mRNA and protein synthesis in the hippocampus

PCR analysis of mRNA for nNOS and iNOS revealed a significant increase in their expression in the hippocampus of KA-treated animals (3 h post-KA) compared to the vehicle control (Fig. 3.2). Immunostaining for nNOS and iNOS revealed that by 3 days post-KA there was an increase in the number of $nNOS^+$ cells (predominantly neurons) and $iNOS^+$ cells (predominantly microglia and astrocytes) compared to control (Fig. 3.2B). The SL and the SLM had greater numbers of nNOS and iNOS cells (Fig. 3.2) compared to other areas of the hippocampus. In addition, in 3 day post-KA treatment there was increased nNOS staining in the neurites, whose cell bodies were located in the outer margins of the pyramidal layer (Fig. 3.7) in the CA3 and CA1 regions suggesting an increase in the local release of NO.

3.3.3 Effect of NOS or sGC inhibition on ADNP expression under basal physiological conditions in the hippocampus

ADNP mRNA and protein was present in the hippocampus as revealed by RT-PCR and immunostaining (Fig 3.3). Cellular localization using cell-specific markers with double/triple immunostaining revealed that ADNP was present in both neurons and glia cells in the CA1 and CA3 regions of the hippocampus (Fig. 3.3B). Interestingly, apical dendrites in the stratum oriens (SO) of the CA1 pyramidal neurons were intensely stained for ADNP (Fig. 3.7). Pyramidal cells in the CA1/CA3 and interneurons/astrocytes in the SO, stratum radiatum (SR) and stratum lucidum (SL) also contained ADNP in their cell bodies (Fig. 3.3, 3.7).

In order to determine whether the NO-cGMP pathway has a role in the physiological expression of ADNP under control conditions in the hippocampus, animals were treated with the NOS or sGC inhibitor (without the induction of seizure by KA). N.B. these animals were controls for the 3 hr post-KA group and were approximately euthanized 30 hr after the initial administration of drug (see Fig. 2.1) but will subsequently be referred to as the '3 hr' time point. ADNP mRNA expression was reduced by L-NAME, 7NI or ODQ compared to controls (DW or DMSO) in the 3 hr group but not in the 3 day control group (these animals were the controls for 3 day post-KA) (Fig. 3.3A). This shows that after a transient block of the NO-cGMP signalling system, ADNP production is inhibited but recovers to control levels within 3 days.

The effect of NOS or sGC inhibition on the topographic distribution of ADNP containing neurons in the CA3 and CA1 regions of the hippocampus was further examined by immunostaining (Fig. 3.3B). In the 3 hr group, the number of $ADNP^+$ neurons in the CA3 region of the hippocampus was significantly reduced with L-NAME compared to the vehicle control (Fig. 3.3B, Fig. 3.4). Similarly, 7-NI or ODQ treatment also caused a reduction in the number of $ADNP^+$ neurons in the CA3 compared to the vehicle (DMSO) group (Fig. 3.3, Fig. 3.4). However, 3 days post drug treatment, there was no significant difference in $ADNP^+$ neuron numbers compared with appropriate vehicle control groups (Fig. 3.4).

3.3.4 Effect of NOS or sGC inhibition prior to kainic acid treatment on ADNP in the hippocampus

Following seizure (KA treatment) a significant reduction of both ADNP mRNA and protein synthesis was observed by 3 days as revealed by RT-PCR/qPCR, immunostaining and Western blot analysis (Fig. 3.5, Fig. 3.6). Immunostaining for ADNP/NeuN/DAPI and TUNEL in the hippocampus from 3 day post-KA animals did not show cell loss/ apoptosis indicating that the reduction in ADNP is not due to cell loss (Fig. 3.9, Fig. 3.10).

The suppression of ADNP mRNA by KA alone (without NOS or sGC inhibitor pre-treatment) was evident in the 3 day post-KA treated animals but not in the 3 hr post-KA group. However, L-NAME, 7-NI or ODQ pre-treatment reduced ADNP mRNA levels in the 3 hr post KA group, compared to appropriate vehicle pre-treatment groups within this group (vehicle + KA) (Fig 3.5, Fig. 3.6). This implies that this initial reduction in ADNP mRNA levels was due to reduced production of NO and cGMP as opposed to the effects of KA (for 3 hr), which correlates with the effects of these drugs seen under basal conditions (Fig. 3.3, Fig. 3.4). Interestingly, by 3 days post-KA treatment, ADNP reduction was reversed in the hippocampus in the NOS or sGC inhibitor pre-treated group but not in the vehicle pre-treated groups (Fig 3.5, Fig 3.6). This indicates that increased NO levels, as evident from increased nNOS and iNOS mRNA expression and immunostaining (Fig. 3.2) following KA treatment (by 3 days), decreases ADNP. ADNP immunostaining of the CA3 and CA1 regions of the 3 day post-KA treated animals, showed that a decrease in ADNP synthesis was evident in both neurons and astrocytes (Fig. 3.6, 3.7). In the control group ADNP was observed throughout the soma and apical dendrites of the CA1 pyramidal neurons (SO), while in the 3 day post-KA treatment group ADNP staining was observed in clusters and/or in punctate form within the nucleus of neurons in the pyramidal layer (SP). ADNP was almost completely suppressed in the apical dendrites in the SO (Fig. 3.7).

3.3.5 Effect of NOS or sGC inhibition prior to kainic acid treatment on onset of the first seizure

Following KA administration, animals were monitored and the symptoms leading to the onset of the first full generalised seizure were scored according to Racine's seizure severity scale (stages 1-5) as described previously. In the KA treated group, without NOS or sGC inhibitor treatment, animals progressed steadily from stage 1 to 5 within 90 mins of KA treatment. In general, L-NAME pre-treated animals exhibited the first full generalised seizure sooner (25 ± 15 min) than animals that had been pre-treated with vehicle control (DW or DMSO), 7-NI or ODQ (70 ± 20 min). This may suggest that the inhibition of all NOS isoforms by L-NAME, advances the onset of KA-induced seizure activity whilst a more selective inihibition does not. It is possible that decreased ADNP expression by L-NAME treatment prior to KA may play a part in this.

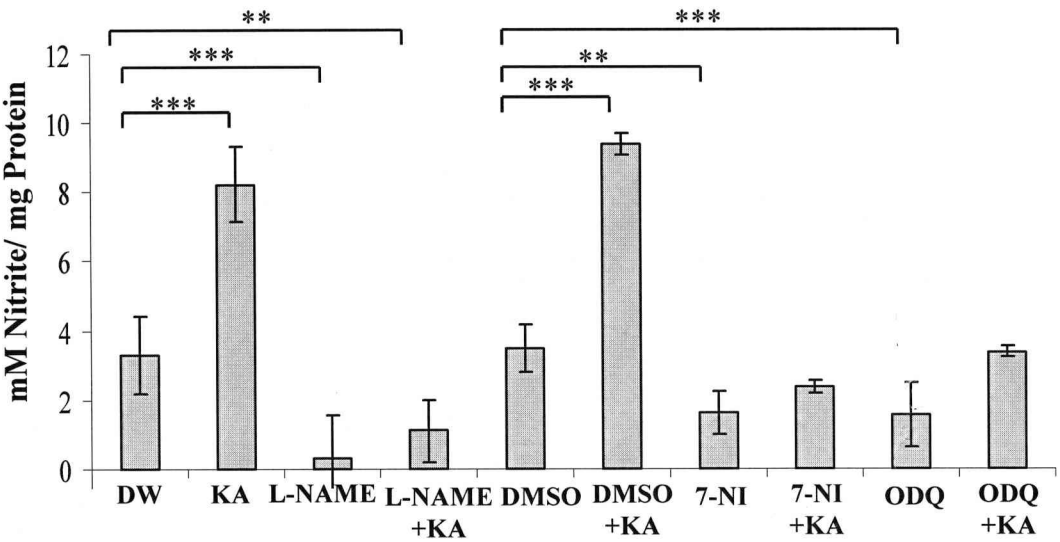


Figure 3.1 The effect of NOS inhibitor L-NAME (50 mg/kg i.p.), neuronal NOS inhibitor 7-NI (50 mg/kg i.p.), sGC inhibitor ODQ (10 mg/kg i.p.) and KA treatment (10 mg/kg i.p.), on nitrite concentration in the hippocampus. Samples were taken after the initial seizure (3 hr group) and show an increase in nitrite levels following seizure, whereas L-NAME, 7-NI and ODQ showed a decrease in nitrite levels (**p>0.01, ***p>0.001 n=3).

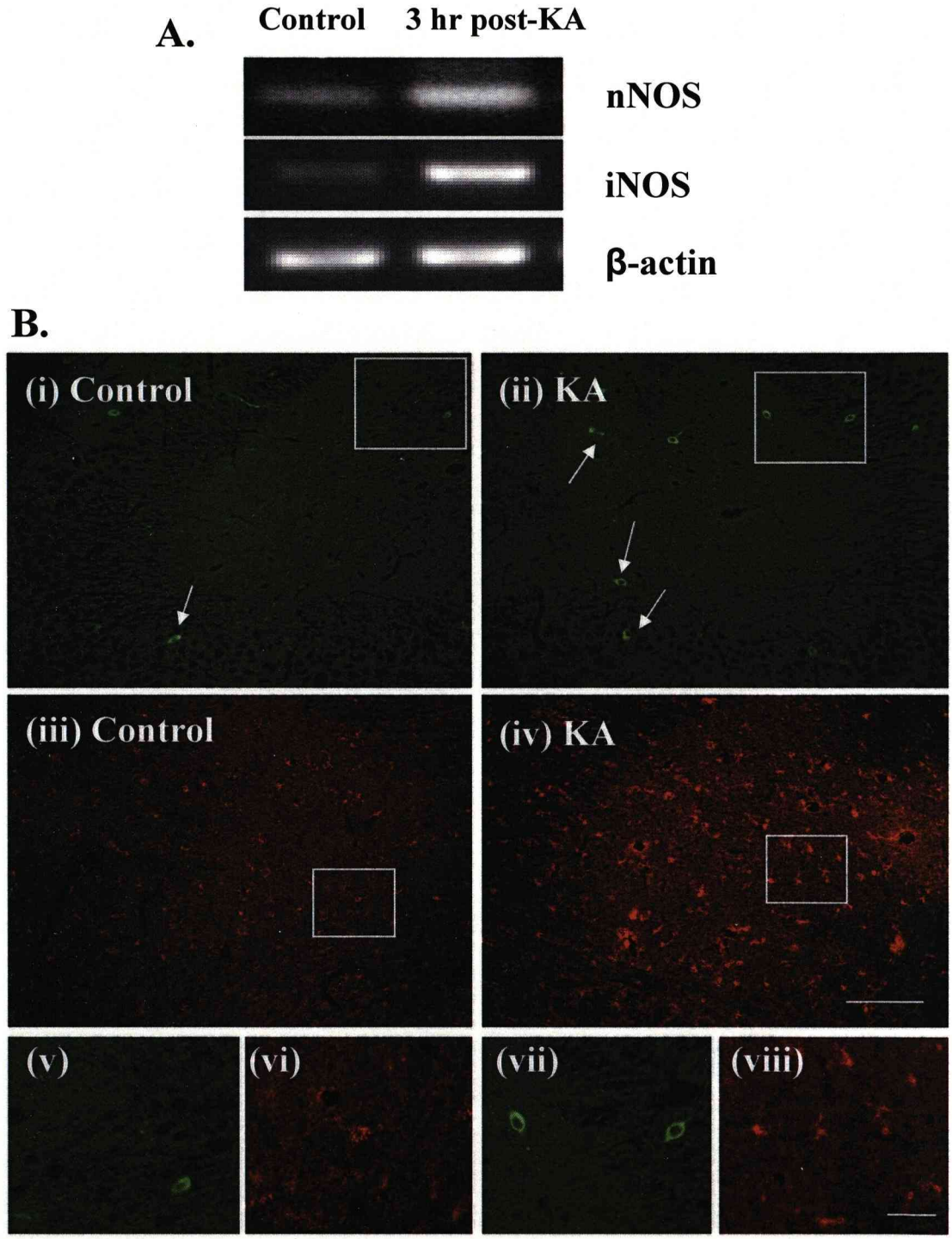


Figure 3.2 Kainic acid (KA)-induced seizures increase NOS expression in the hippocampus. A. RT-PCR analysis and B. immunocytochemistry for nNOS and iNOS show that KA increases gene transcription by 3 hr after KA treatment (A) and protein synthesis by 3 days (B). $nNOS^+$ (green) cells indicated by arrows in (i) and (ii) represent $nNOS^+$ neurons and red cells in (iii) are $iNOS^+$ (predominantly glia). (v) and (vi) represent higher magnification views of $nNOS^+$ and $iNOS^+$ cells denoted by the box in (i) and (iii) respectively. Note that there is an increase in nNOS and iNOS cells in KA-treated animals compared to vehicle control. Scale bar: 100 μm (i)-(iv), 25 μm (v)-(viii).

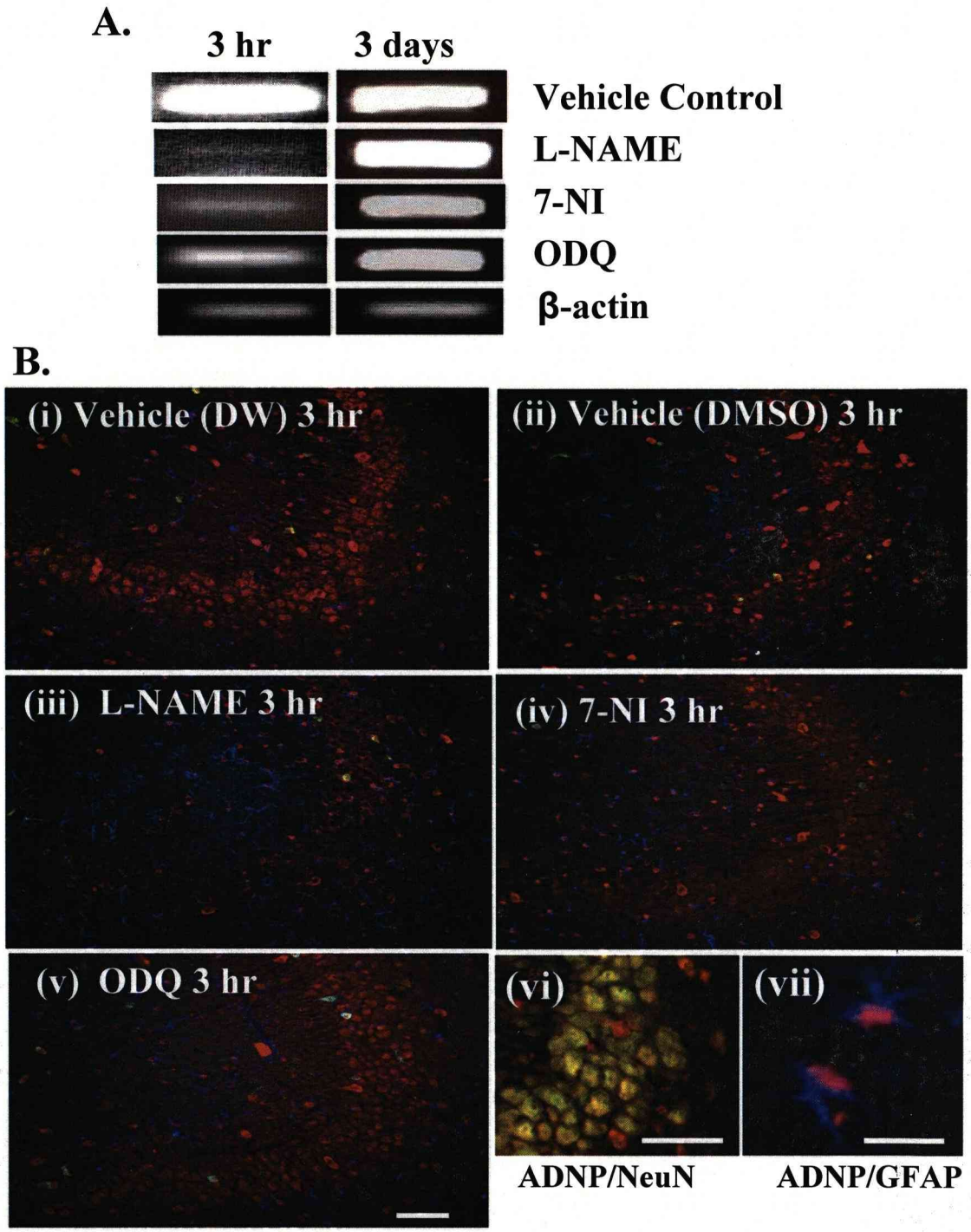


Figure 3.3 Effect of NOS or sGC inhibition on ADNP in the hippocampus *in vivo*. A. ADNP mRNA expression. L-NAME, 7-NI and ODQ reduced the amount of ADNP mRNA in the 3hr group compared with control. Three days after treatment, ADNP mRNA levels returned to basal levels. B. Immunostaining. Effect of NOS or sGC inhibition (3 hr group) on ADNP+ (red), nNOS+ (green, except in vi) and GFAP+ (blue) cells in the CA3 region. Vehicle control treatments (i) DW and (ii) 10% DMSO had no effect on ADNP staining or distribution. The 3 hr group showed that with L-NAME treatment (ii) ADNP was markedly reduced in the CA3 and a smaller reduction was seen with 7-NI (iv) and ODQ (v) treatment. At higher magnification, ADNP co-immunostained in both neurons (vi, green cells are NeuN+) and in astrocytes (vii, blue cells are GFAP+). Scale bar: 100 μm (i-v), 50 μm (vi). 20 μm (vii).

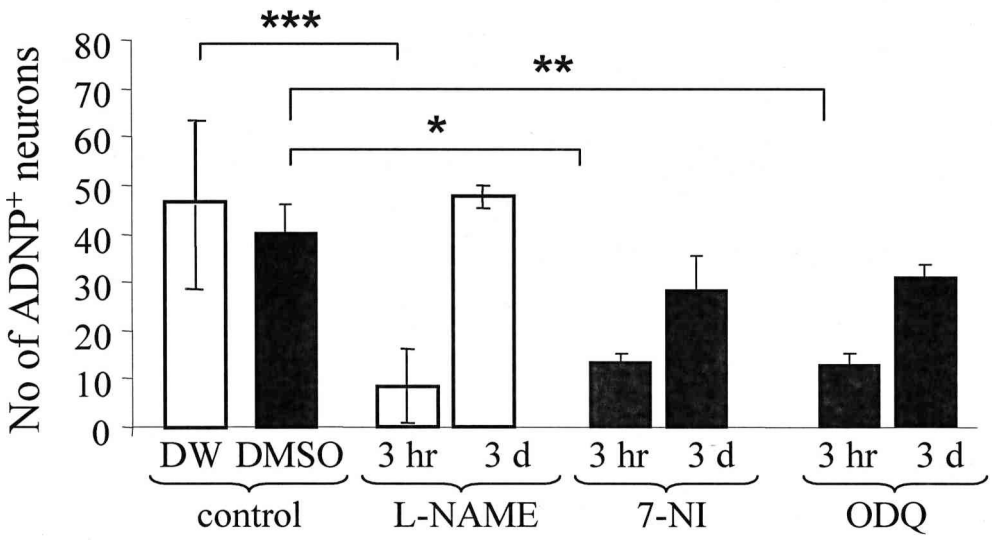


Figure 3.4 Quantification of $ADNP^+$ neurons in the CA3 following NOS and sGC inhibition. In the 3 hr group, NOS or sGC inhibitor treatment, caused a significant reduction in the number of $ADNP^+$ neurons compared with the appropriate control (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$; $n=3$). By 3 days the number of $ADNP^+$ neurons had nearly returned to basal levels.

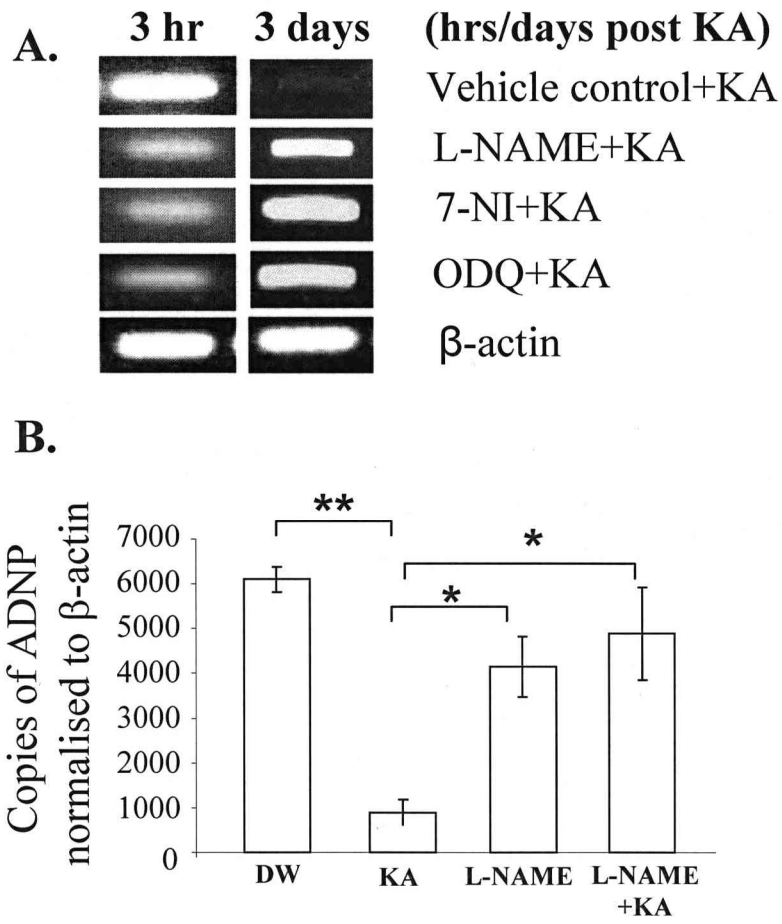
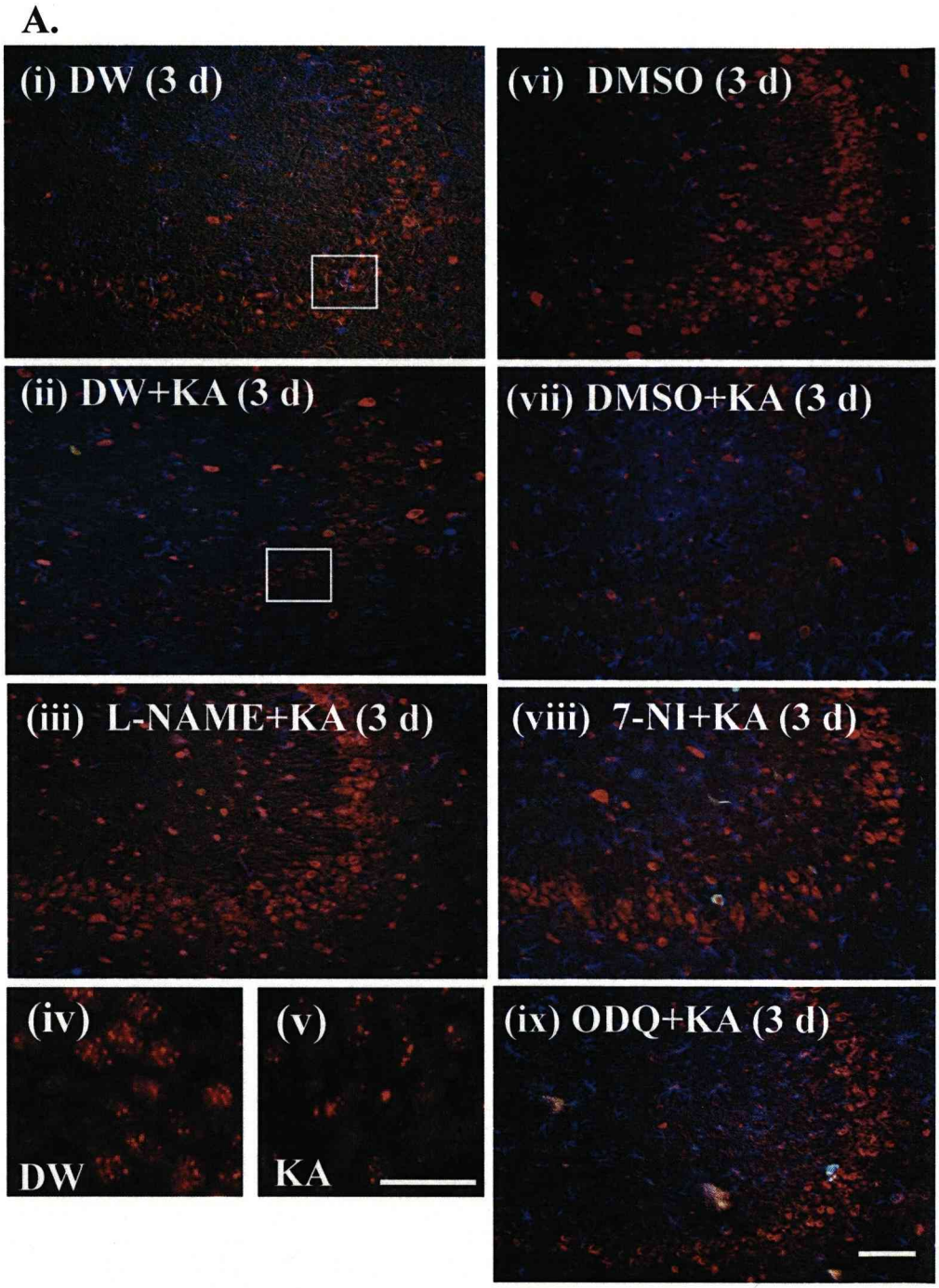


Figure 3.5 KA-induced seizure significantly reduces ADNP mRNA expression 3 days. Pre-treatment with the NOS inhibitors, L-NAME/7-NI or the sGC blocker, ODQ reverses the effects of KA-induced ADNP suppression. RT-PCR (A) and qPCR (B) analysis of ADNP mRNA revealed that KA suppresses ADNP mRNA by 3 days post KA treatment, but not by 3 hrs (A). Treating with 7-NI or ODQ prior to KA reverses ADNP mRNA levels to nearly basal levels by 3 days.


A.
(i) DW (3 d)
(vi) DMSO (3 d)
(ii) DW+KA (3 d)
(vii) DMSO+KA (3 d)
(iii) L-NAME+KA (3 d)
(viii) 7-NI+KA (3 d)
(iv)
DW
(v)
KA
(ix) ODQ+KA (3 d)

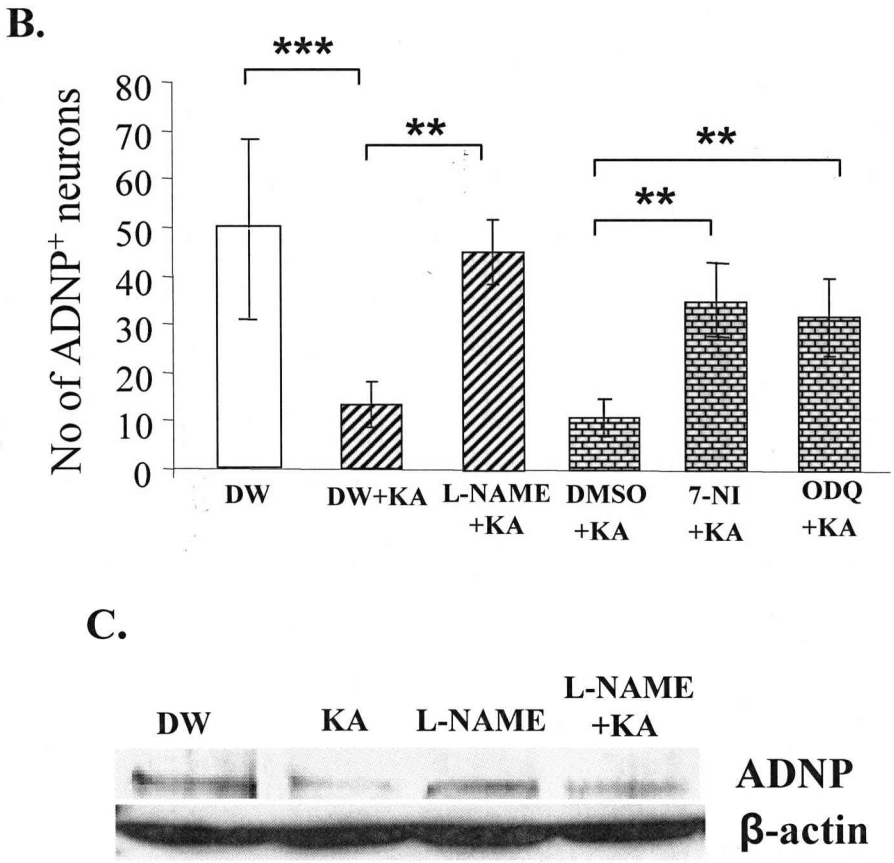


Figure 3.6 KA-induced seizure significantly reduces ADNP expression in the hippocampus by 3 days. A Immunostaining in the CA3 treated with appropriate vehicle control (i, DW and vi, DMSO), KA treatment following these controls (ii, DW+KA and vii, DMSO+KA) produced a significant decrease in ADNP$^+$ cells by 3days. Pre-treatment with the NOS or the sGC inhibitors (iii, L-NAME +KA, viii, 7NI+KA, ix, ODQ+KA) reversed this effect. The higher magnification images show in more detail a decrease in ADNP expression in the soma of CA3 neurons after KA treatment (v) compared with vehicle control (iv) (the areas correspond to the boxed regions in i and ii.). Scale bar: 100 μm (i-iii and v-ix) and 20 μm (i and ii) B. Quantification of ADNP$^+$ neurons. KA treatment for 3 days significantly decreased the number of ADNP$^+$ neurons in the CA3 region compared with the appropriate control and pre-treatment with NOS or sGC inhibitors reversed KA-induced ADNP reduction (*p <0.05, **p <0.01, ***p <0.001; n = 3).
C. Western blot analysis of ADNP from the whole hippocampus also reveals a decrease ADNP protein following KA treatment which is partially reversed by pre-treatment with L-NAME.

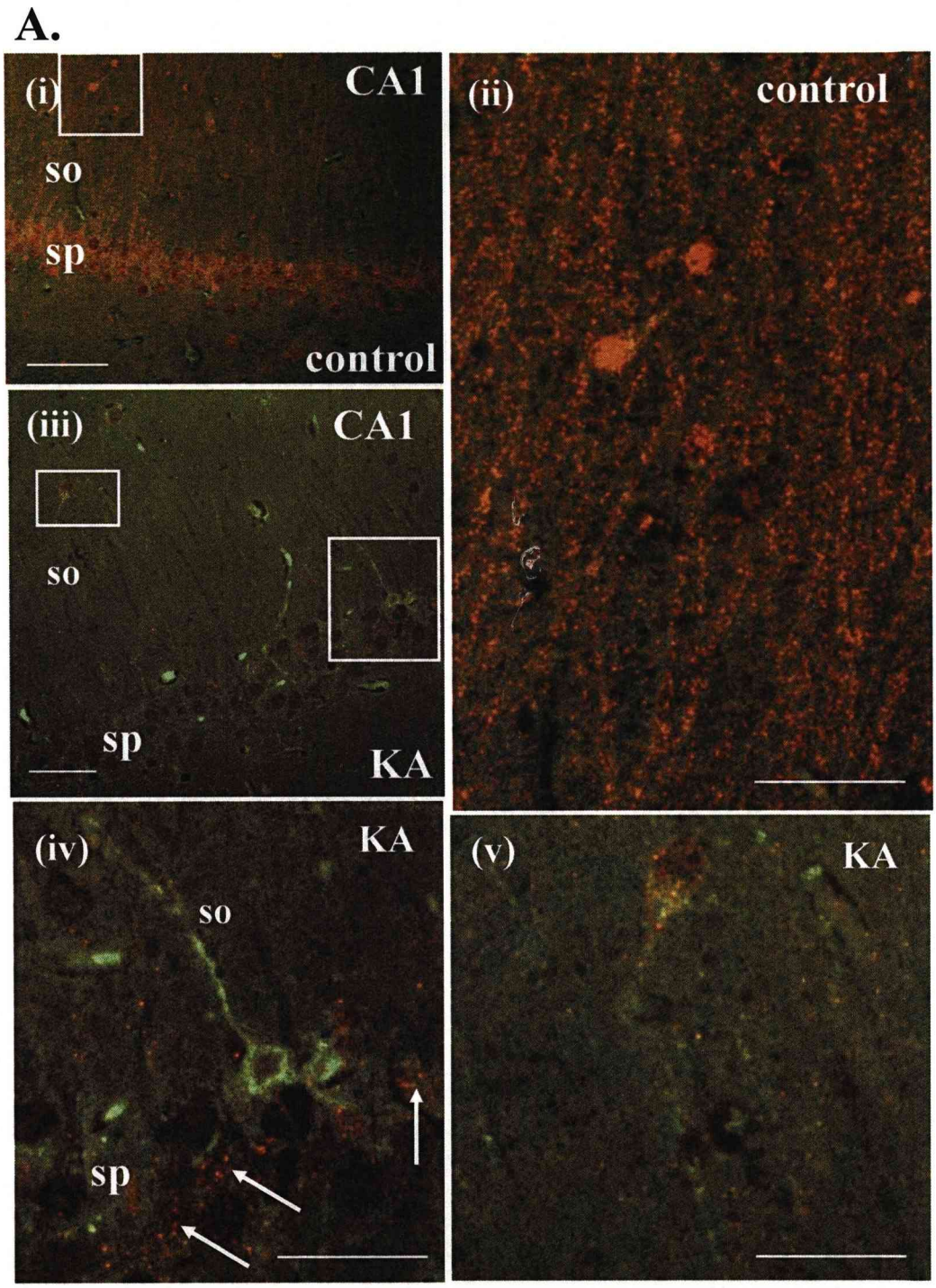


Figure 3.7 Effect of KA and NOS or sGC inhibition on ADNP in the CA1 region of the hippocampus in vivo.A. Immunostaining. (i) In vehicle control immunostaining of ADNP is seen in the cell bodies of CA1 pyramidal neurons in the stratum pyramidale (sp). (ii) Higher magnification of the area shown by the box in (i), shows intense ADNP staining in apical dendrites of the pyramidal neurons traversing the stratum oriens (so). (iii) KA-induced epilepsy increases the number of $nNOS^+$ cells (green) whilst dramatically reducing $ADNP^+$ (red) in the pyramidal cells of CA1 region of the hippocampus. (iv) Increased nNOS is primarily in the soma and dendrites of neurons. ADNP is confined mainly to the nucleus of a few neurons in the stratum pyramidale (sp). (v) Higher magnification of the area indicated by the box in iii (in the 'so' region) shows the loss of ADNP staining from the apical dendrites and reveals nNOS staining in some fibres. Scale bar: 100 μm (i) and (iii), 20 μm (ii, iv and v).

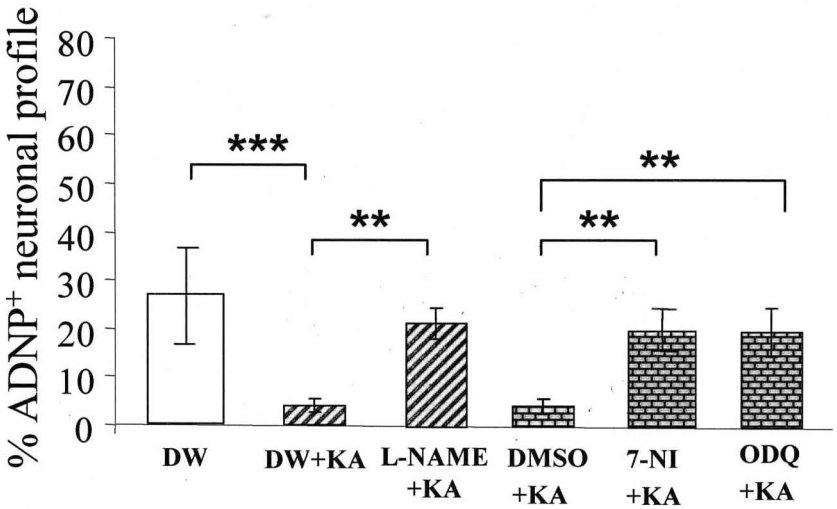


Figure 3.8 ADNP$^+$ neuronal quantification in the CA1 region of the hippocampus in vivo with NOS and sGC inhibitors 3 days after KA treatment. ADNP$^+$ neurons were counted from the CA1 stratum pyramidale. In controls, ADNP expression was reduced by 3 days following KA treatment. Almost complete reversal was seen with NOS or sGC inhibitor pre-treatment. *p <0.05, **p <0.01, ***p <0.001; n=3.

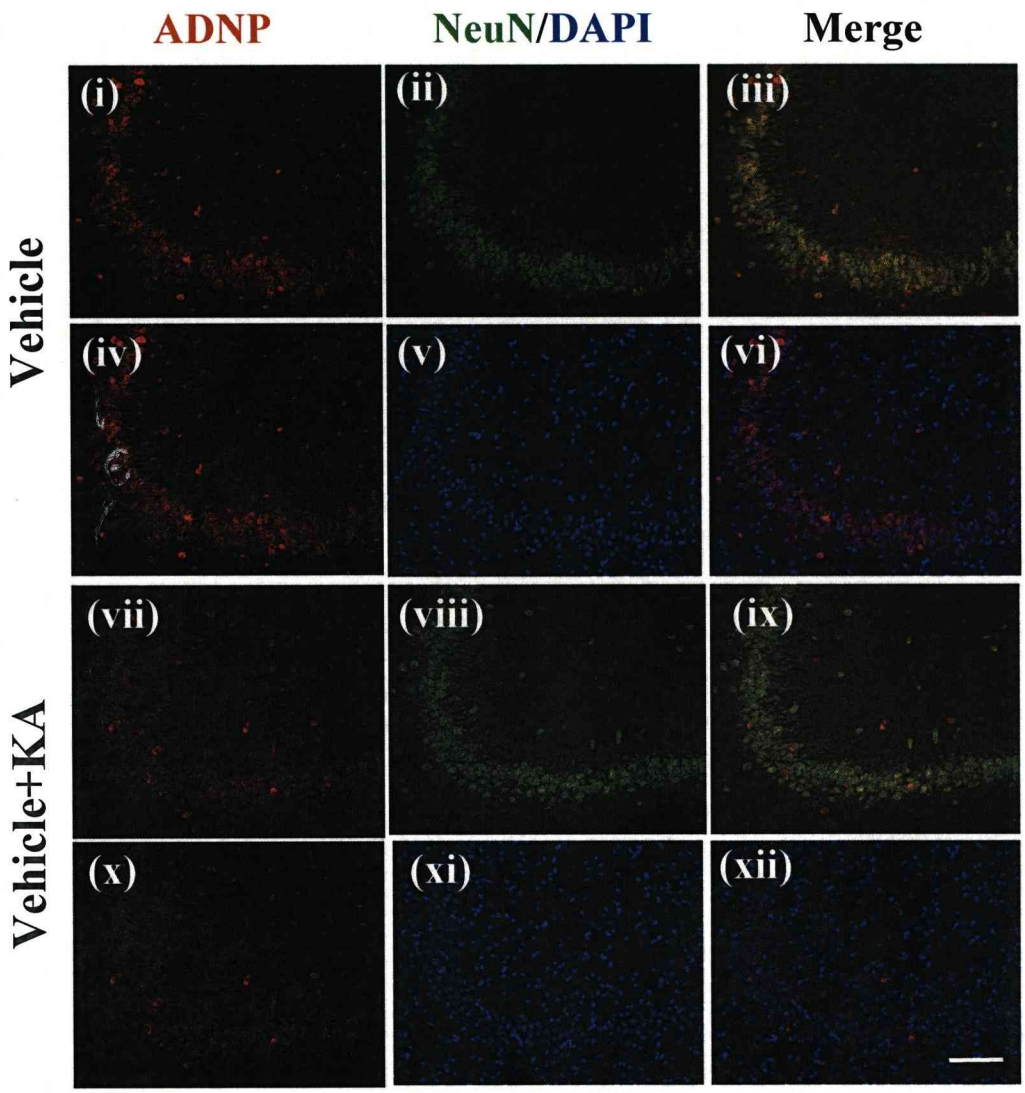


Figure 3.9 CA3 region of the hippocampus from vehicle control (i-vi) and vehicle+KA treated (vii-xii) animals (3 day group) stained for ADNP (red), NeuN (green) and DAPI (blue). Merged images (iii, vi, ix and xii) show co-immunostaining of $ADNP^{+}$ cells NeuN/DAPI. NeuN and DAPI staining show the presence of neuronal cells/nuclei in the CA3 following KA, suggesting that a decrease in ADNP expression in the hippocampus is not due to cell loss. Scale bar: 100μm.

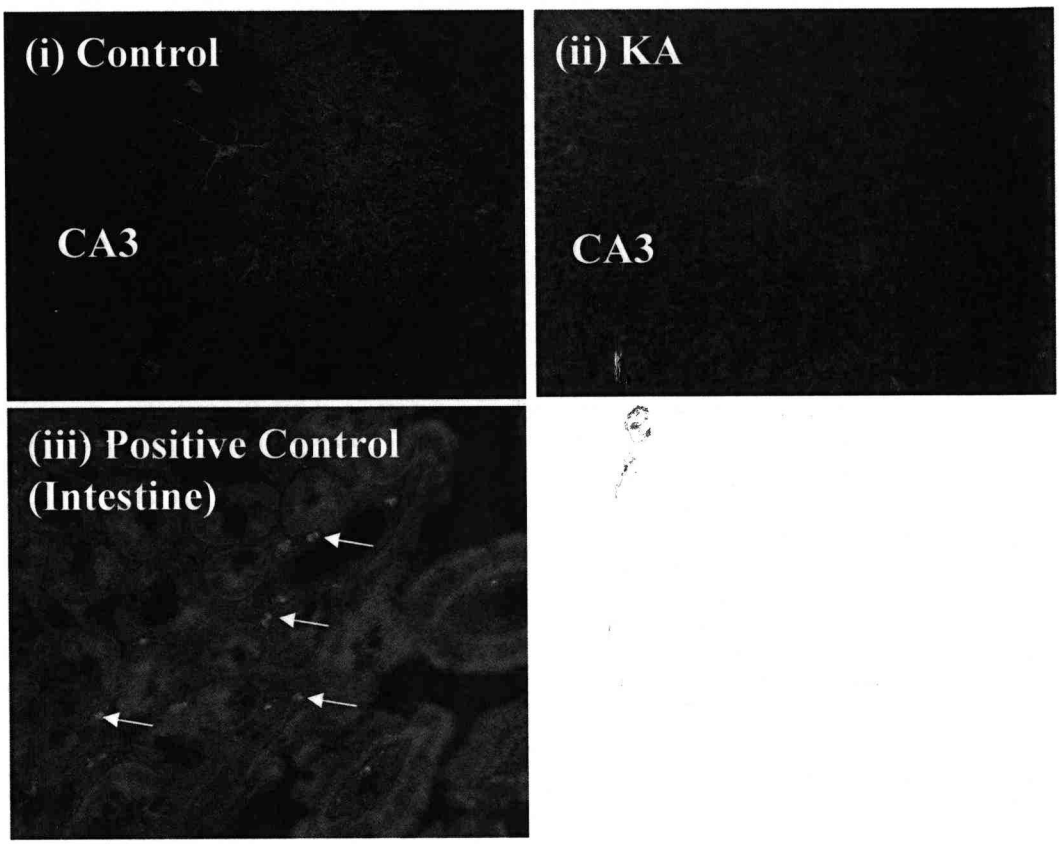


Figure 3.10 TUNEL staining in hippocampus (CA3) of vehicle control and KA treated animals. Lack of TUNEL$^+$ cells in the CA3, suggest that that a decrease in ADNP expression in the CA3 is not due to cell loss. (iii) shows positive control tissue for TUNEL$^+$ staining carried out alongside sections shown in (i) and (ii) Arrows in (iii) indicate TUNEL$^+$ cells.

3.4 Discussion

The neuroprotective role for VIP-responsive ADNP and its derivative peptides, such as NAP, have previously been discussed (Gozes, 2007). Although studies of ADNP in experimental epilepsy have not been reported VIP does seem to have a role. VIP levels fall acutely in experimentally induced seizures (Romualdi et al., 1992), whilst post-mortem hippocampus samples from human TLE have increased VIP binding (De Lanerolle et al., 1995). Initial studies of ADNP suggested that it was primarily located in glia cells (Gozes, 2007). Previously, Bassan et al. (1999) showed increased basal levels of ADNP mRNA in astrocytes in response to VIP and, interestingly, VIP and/or its derivative peptides induce NO and cGMP production in cortical cultures (Ashur-Fabian et al., 2001). An important role for NO in neuron-glia communication and neuroprotection has previously been reported (Thippeswamy et al., 2005, 2007a). It is thus plausible that NO could be involved in regulating VIP-responsive ADNP function. These results show that ADNP was detectable under basal conditions in the pyramidal neurons and interneurons of the hippocampus in addition to astrocytes. The antibodies used were raised against the peptide sequence 989-1015 of the human ADNP homologous to the rat and mouse sequence and does not share homology with other proteins in a BLAST search. Interestingly, ADNP staining in the apical dendrites of the pyramidal cells in the SO was intense under basal conditions, suggesting large amounts of the protein under basal conditions (Fig. 3.7A), but was conspicuously absent in the SL and mossy fibres. This reveals a highly selective distribution indicating a cell-specific, as opposed to, a generalised function.

At the doses used in these experiments, KA initially activates kainate receptors in the hippocampus (Ben-Ari and Cossart, 2000) but this rapidly leads to more widespread neuronal depolarisation and release of transmitters such as glutamate acting on both AMPA and NMDA receptors. The neuron depolarisation and NMDA channel activation leads to calcium influx resulting in activation of NOS and subsequent NO production (Kato et al., 2005). NO released from nNOS activates sGC to produce cGMP in many areas of the brain including the hippocampus (Garthwaite et al., 1989; Teunissen et al., 2001). In this chapter the effects of NO and cGMP on ADNP distribution and synthesis

were examined in the presence and absence of NOS and sGC inhibitors in the hippocampus. The sGC inhibitor, ODQ was used to inhibit cGMP production to understand whether ADNP is also regulated by the sGC-cGMP pathway. Several others have used these NOS or sGC blockers, in vivo, via the intraperitoneal route and have demonstrated that they cross the blood brain barrier (Bagetta et al., 2002; Catania et al., 2003; Gupta and Dettbam 2003; Kato et al., 2005; Chuang et al., 2007; Liu et al., 2007; Parathath et al., 2007). The decreased nitrite content in the hippocampus of animals treated with NOS or sGC inhibitor confirmed that the drugs were effective in inhibiting NO and cGMP production (Fig. 3.1).

KA treatment significantly reduced ADNP mRNA and protein synthesis by 3 days following seizure, but not by 3 hrs suggesting a complex pathway involving KA-induced ADNP suppression. Immunostaining with the neuronal marker NeuN and TUNEL staining for apoptosis in the hippocampus from these animals confirmed that there was no cell loss (Fig. 3.9, Fig. 3.10). The delay in suppression of ADNP synthesis coupled with the later loss of ADNP staining in the CA regions studied could result from increased NO production by KA-induced seizures. The 3 day post-KA group were treated with diazepam after the initial seizure to prevent SE and mortality. It has been previously demonstrated by Du et al. (1995) that diazepam rescues neurons from calcium-induced death in a KA model of epilepsy. This, coupled with the lack of cell death seen in our model, infer that the decrease in ADNP mRNA and protein in KA-treated animals is not merely a manifestation of cell loss. Diazepam also limits the severity of seizures (Pitkänen et al. 2005) and this would be predicted in turn to reduce excessive NO production, thus reducing the neurotoxic actions of NO (Rajasekaran, 2005; Chuang et al., 2007). In the present study, the animals that received diazepam and NOS or sGC inhibitors showed complete reversal of ADNP reduction 3 days after KA treatment. These results suggest that the use of antiepileptic drugs combined with NOS inhibition may be beneficial (Paul, 2003; Luszczki et al., 2006).

To elucidate the precise mechanism of KA-mediated ADNP suppression by NO will require more detailed research. However, following KA treatment it was noted that

ADNP was mainly located in the nuclei of the pyramidal neurons while in the animals pre-treated with the NOS blocker, it was distributed throughout the soma and dendrites. As ADNP is synthesised in the cytoplasm, the presence of peptide in the nucleus is indicative of translocation and it is possible that ADNP may function as a DNA binding protein/transcription factor to regulate gene expression. Zemlyak et al (2007) have recently shown that the ADNP-derivative, NAP protects KA-treated hippocampal neurons in culture by interacting with microtubule-associated protein (MAP).

Increased NO concentration in the CNS causes DNA damage, altered mitochondrial membrane potential and activation of polyADP-ribosylation leading to neuronal death (Zhang et al., 1994; Dawson and Dawson, 1995; Wallis et al., 1996). Depending on the NO levels, it can modulate DNA-binding activities of the cAMP response element-binding protein (CREB, a transcription factor) to mediate the cGMP-PKG-dependent anti-apoptotic signals induced by NO (Nagai-Kusuhara et al., 2007; Zhuravliova et al., 2007; Riccio et al., 2006). Interestingly another VIP-derived peptide, ADNF-9, also protects neurons from iNOS-mediated toxicity in the hippocampus in a hypoxic-ischemia model (Kumral et al., 2006). Low concentrations of NO found during basal conditions are known to be beneficial (Contestabile and Ciani, 2004; Thippeswamy et al., 2006; Calabrese et al., 2007). The reduction of ADNP produced by the inhibition of NOS or sGC (in the absence of seizure) suggests that the basal production of NO may be important for maintaining ADNP synthesis in the hippocampus. Based on these observations it is plausible that ADNP and NO may interact to regulate neuronal survival. Recent studies have shown that NO interacts with ADNP downstream targets. Recently, Stroissnigg and colleagues demonstrated that MAP can undergo S-nitrosylation and that this is involved in regulation of growth cones morphology (Stroissnigg et al., 2007). In the present study, KA-induced NO suppression of ADNP in apical dendrites of the pyramidal neurons may also suggest an NO-ADNP interaction in orchestrating the onset morphological following seizure.

In summary, during physiological conditions, a low concentration of NO appears to promote ADNP synthesis since the inhibition of NO decreases ADNP and advances the

onset of the first seizure in the KA-induced model of epilepsy. KA increases NO production in the hippocampus and these increased levels of NO may suppress ADNP. Treating animals with the NOS or sGC inhibitor prior to KA reverses ADNP suppression by 3 days post-KA treatment, implying its regulation via the NO-cGMP pathway.

CHAPTER 4

The Regulation of ADNP via the NO-cGMP Pathway in the Dentate Gyrus Following Seizure

4.1 Introduction

The dentate gyrus (DG) forms part of the hippocampal formation and plays a vital role in learning and memory. More recently, it has become the subject of extensive research attributable to it being one of the few sites to display postnatal neurogenesis (Kuruba & Shetty, 2007; Taupin, 2006). Distinct neuronal populations in the dentate gyrus, for example hilar neurons, display differential vulnerability to various forms of insult such as traumatic brain injury, ischaemia and epilepsy (Lowenstein et al., 1992; Jiao and Nadler, 2007). The processes following seizure activity and the subsequent fate of cells in the DG are still to be discerned. However, analysis of genes expressed in the DG during development and epileptogenesis suggest that genes involved in neurite outgrowth and neurogenesis are involved (Elliot et al., 2003). ADNP and NAP have been shown to modulate polyADP-ribosylation (PARP) to promote neuronal differentiation and survival via MAPK-PI_3K/Akt pathways (Mandel et al., 2007; Mandel & Gozes 2007; Pascual & Guerri, 2007), similar to the effect caused by NGF on PC12 cells (Visochek et al., 2005). In addition, the genes involved in cell survival or death and plasticity in the DG, for example, neurotrophins are also differentially expressed during epileptic conditions (Sutula et al., 2003; Soriano et al., 2006; Walker, 2007) suggesting the relevance of the neurotrophic actions of VIP/ADNP in seizure.

We and others have shown that following kainic acid (KA)-induced seizure both inducible NOS (iNOS) and neuronal NOS (nNOS) are upregulated (Chuang et al., 2003; Cosgrave et al., 2008; Zaja-Milatovic et al., 2008). NO generates cGMP in many areas of the brain including the hippocampus which can activate transcription factors (Garthwaite et al., 1989; Teunissen et al., 2001; Matsumura et al., 2008). The ADNP sequence contains a homeobox domain, which is a DNA binding domain, many homeotic transcription factor genes are essential during development which ADNP has been shown to be involved in (Mandel et al., 2007). The presence of zinc finger domains in the ADNP gene sequence and the homeobox domain suggest that ADNP may act as a transcription factor.

ADNP has been expression in the DG has not yet been characterised, however, in the hippocampus VIP is contained in the synapses of hilar interneurons which are resistant to seizure-induced damage (Sloviter, 1987). In the previous chapter we demonstrated that ADNP synthesis was significantly suppressed in the CA3 and CA1 pyramidal neurons of the hippocampus following KA-induced seizure in rats which could be reversed if they were pre-treated with NOS or sGC inhibitor (Cosgrave et al., 2008). This implies that the NO-cGMP pathway regulates ADNP synthesis in the hippocampus. Following on from these observations this chapter will investigate the role of the NO-cGMP pathway in the regulation of ADNP expression in different layers of the DG following seizure.

4.2 Materials and Methods

Materials and methods were carried out as previously described for in vivo injections of NO inhibitors and KA. Tissues were processed for immunofluorescence, PCR, qPCR as described in the methods section.

4.3 Results

4.3.1 Effect of NOS or sGC inhibition on ADNP expression under basal physiological conditions in the dentate gyrus

Immunostaining using cell-specific markers revealed that ADNP was present in both astrocytes and neurons in the DG (Fig. 4.1, Fig 4.10) and that ADNP was uniformly distributed in the cytoplasm of the cell bodies of neurons and astrocytes. Astrocytic processes did not show ADNP. The apical dendritic processes of the granule cells in the SG that projected into the outer molecular layer (OML) contained ADNP but the distal processes in the hilus did not. Polymorphic cells in the subgranular zone (SGZ) and interneurons and astrocytes in the hilus were immunostained for ADNP (Fig. 4.1).

In order to investigate whether the NO-cGMP pathway has a role in the physiological expression of ADNP under basal conditions in the DG, animals treated with the NOS or sGC inhibitor without the induction of seizure with KA were examined. ADNP mRNA analysis of the DG and immunostaining for ADNP did not reveal a significant change in

7-NI or ODQ treated animals compared to appropriate controls (Fig. 4.2). In contrast, the broad spectrum NOS inhibitor, L-NAME caused a reduction in the number of $ADNP^+$ cells both in the 3 hr and 3 day groups (Fig. 4.2, Fig. 4.3).

The effect of L-NAME on the topographic distribution of $ADNP^+$ cells in different layers of the DG was further examined by immunostaining. ADNP staining was suppressed in the granule cells of the SG in the L-NAME 3 hr group (Fig. 4.1, Fig. 4.3, and Fig. 4.11) and in the vast majority of neurons in the hilus (Fig. 4.10, Fig. 4.11). Interestingly, ADNP in the SGZ and the apical dendrites of granule cells in the OML were not affected. In the 3 day group, ADNP staining was lost from the apical dendrites of granule cells in the OML (Fig. 4.1(v)).

4.3.2 Effect of NO inhibition on ADNP expression in the dentate gyrus 3 hours post-KA treatment

KA on its own had no significant effect on ADNP mRNA expression by 3 hrs but pre-treatment with L-NAME significantly increased its expression (Fig. 4.4). L-NAME alone also did not change ADNP mRNA expression levels. In the 3 hr post-KA group that were pre-treated with L-NAME, immunostaining for ADNP revealed a significant increase in $ADNP^+$ granule cells in the SG and hilar neurons when compared with appropriate control (Fig 4.5(iv), Fig. 4.6). RT-PCR and qPCR of the DG also showed an increase in mRNA expression by 3 hrs in L-NAME+KA treated animals (Fig. 4.4). Both KA and L-NAME on their own suppressed ADNP in granule cells in the SG by 3 hrs (Fig. 4.5, Fig. 4.6). L-NAME suppressed ADNP in hilar neurons (Fig. 4.5 (ii), Fig. 4.11), while KA did not (Fig. 4.5(iii), Fig. 4.11) for the 3hr group. Analysis of $ADNP^+$ cell counts from different layers of the DG showed that KA alone caused a slight decrease in $ADNP^+$ cells in the OML and in the SG, but had no effect on the SGZ and hilar cells (Fig. 4.6, Fig. 4.11B) compared with appropriate controls for the 3 hr group. L-NAME treatment prior to KA resulted in a significant increase in the number of $ADNP^+$ granule cells in the SG compared with control (Fig. 4.6).

4.3.3 Effect of NO inhibition on ADNP expression in the dentate gyrus of 3 days post-KA treatment

Interestingly, in the 3 day post-KA group ADNP mRNA and protein synthesis decreased in the L-NAME pre-treated group compared with L-NAME on its own or vehicle (Fig. 4.7). Analysis of ADNP immunostaining of DG layers of L-NAME pre-treated rats (that received KA) further revealed 3 main differences: an increase in the number of $ADNP^+$ cells in the SGZ (Fig. 4.8(iv), Fig. 4.9), a decrease in the number of $ADNP^+$ neurons in the hilus (Fig. 4.10, Fig 4.11) and a decrease in the number of $ADNP^+$ granule cells in the SG (Fig 4.8, 4.9). L-NAME alone also suppresses ADNP in granule cells in the SG compared to vehicle controls (Fig 4.8, 4.9). In L-NAME pre-treated, 3 d post-KA group that were double stained for NeuN and ADNP, hilar neurons showed an eccentric nucleus with an irregular cell boundary (Fig. 4.10 (vi), (b), (c)) compared with a centrally placed nucleus and smooth cell boundary in controls (Fig. 4.10(v), (a)). Hilar neuron counts ($NeuN^+$) from sections representing approximately the same region of the DG in 3 d post-KA group (Fig. 4.10), KA caused a small reduction in the number of $NeuN^+$ neurons compared with vehicle control (Fig. 4.11). 7-NI and ODQ did not cause any significant change in ADNP expression in the DG of normal rat brain or KA-treated animals by 3 hrs or 3 days.

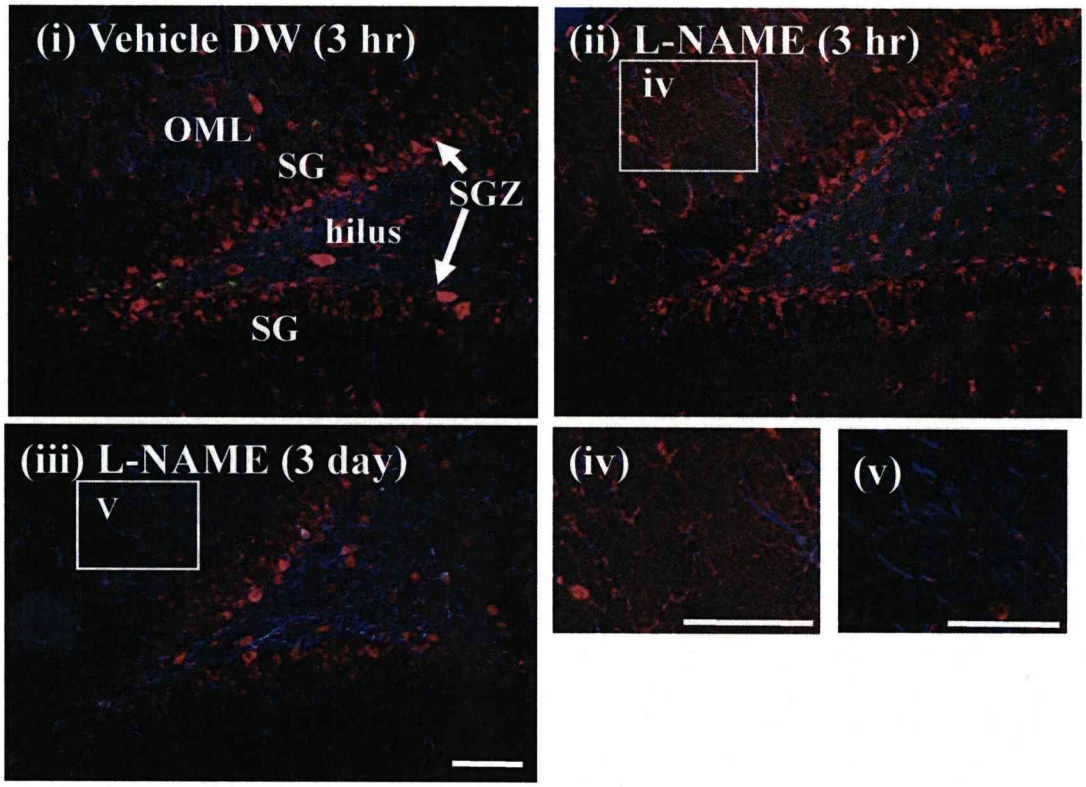


Figure 4.1 Effect of L-NAME treatment on ADNP$^+$ cells in different layers of the DG. All sections were immunostained for ADNP (red), GFAP (blue) and nNOS (green). (i) Different layers of the dentate gyrus: OML, outer molecular layer; SG, stratum granulosum; SGZ, subgranular zone. Large cells (neurons) in the hilus are ADNP$^+$ and some small nNOS$^+$ neurons were located close to the SGZ and in the outer molecular layer. Some ADNP$^+$ neurons in the hilus also co-immunostain with nNOS. (ii) L-NAME treatment in the 3 hr group suppresses ADNP in the granular cell layer and neurons in the hilus. However, nerve fibres in the OML were intensely stained for ADNP compared to vehicle control. (iv) Represents a higher magnification of the area indicated by a box in (ii) showing intense ADNP$^+$ nerve fibres (red). (iii) L-NAME treatment, continues to suppress ADNP in granule cells by 3 days, however, ADNP expression returned in hilar neurons by 3 days. The neurites in the OML were not ADNP$^+$. (v) Represents a higher magnification of the area indicated by a box in (iii)- nerve fibres in the OML show no staining for ADNP. Scale bar: 100 μm.

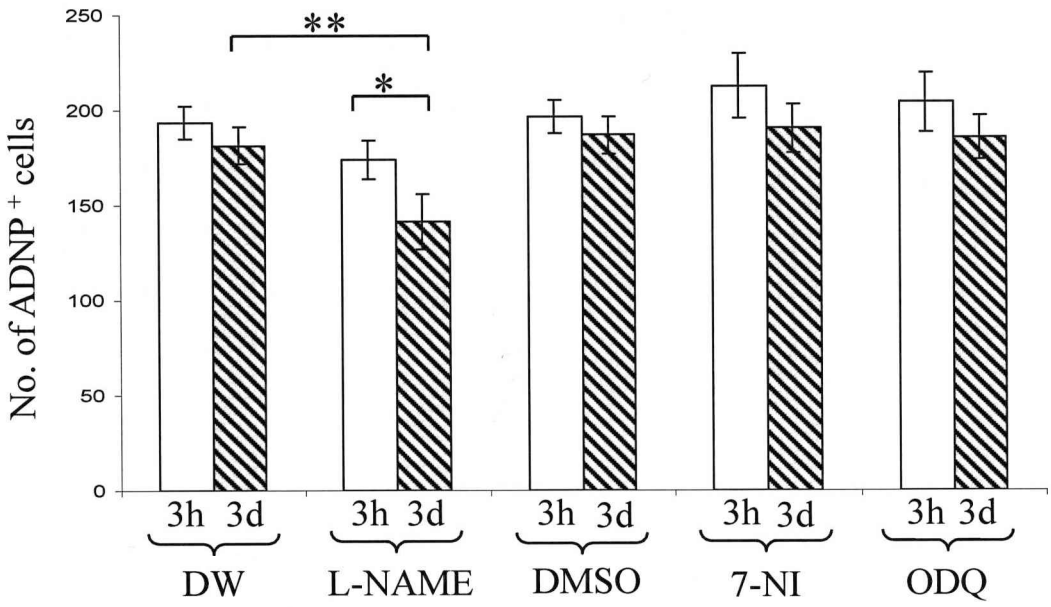


Figure 4.2 Quantification of ADNP$^+$ cells (neurons and astrocytes) from the DG of animals treated for the 3 hr and 3 day control group (without KA) with vehicle control (DW or 10%DMSO, NOS or sGC inhibitor). The neuron specific NOS inhibitor, 7-NI and sGC inhibitor, ODQ had no effect on the overall ADNP$^+$ cell numbers compared with their vehicle control, DMSO. However, L-NAME caused a significant reduction in the number of ADNP+ cells by 3 days compared to vehicle control and the 3 hr group (* $p < 0.05$, ** $p < 0.01$; n=3).

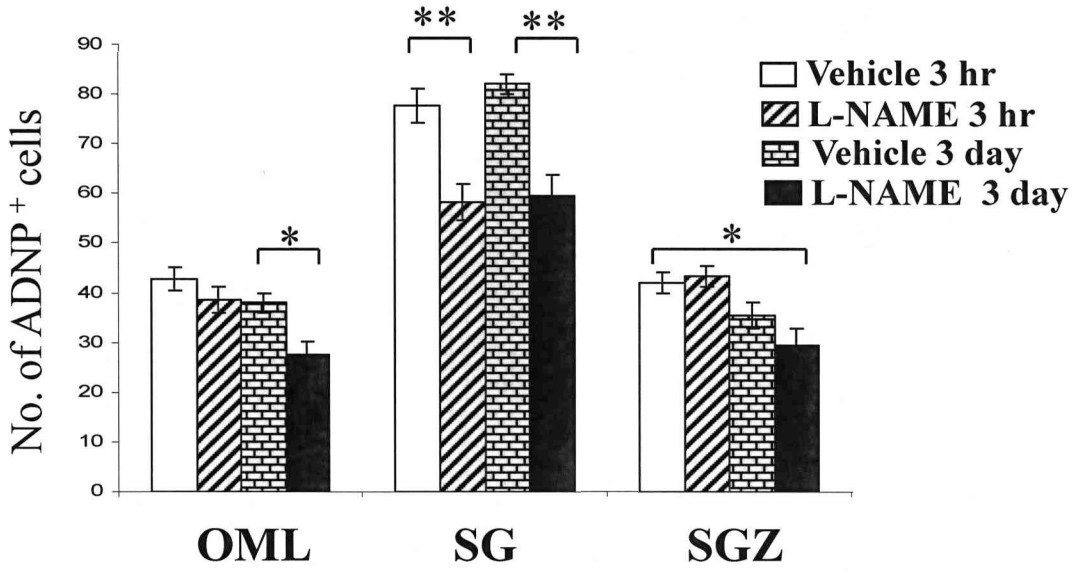
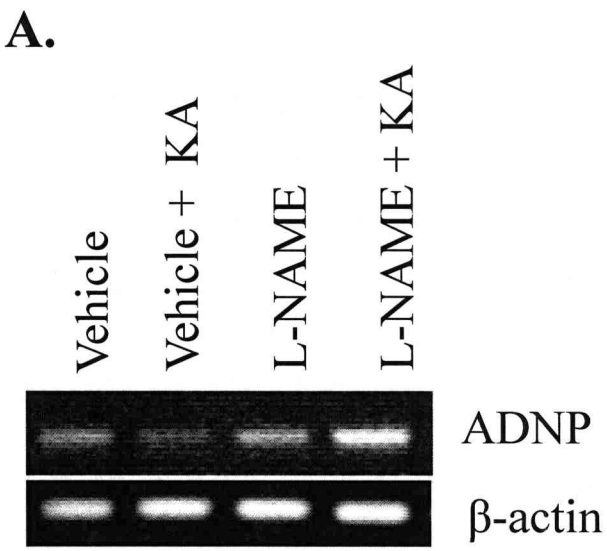


Figure 4.3 Cell counts (ADNP+ neurons and astrocytes) from different layers of the DG from the 3 hr and 3 day control groups. A significant reduction in ADNP$^+$ cells in the SG was seen by 3 hrs with L-NAME and was still evident by 3 days. ADNP$^+$ cell numbers were also reduced in the OML and SGZ in the 3 d group. (* $p < 0.05$, ** $p < 0.01$; n=3).



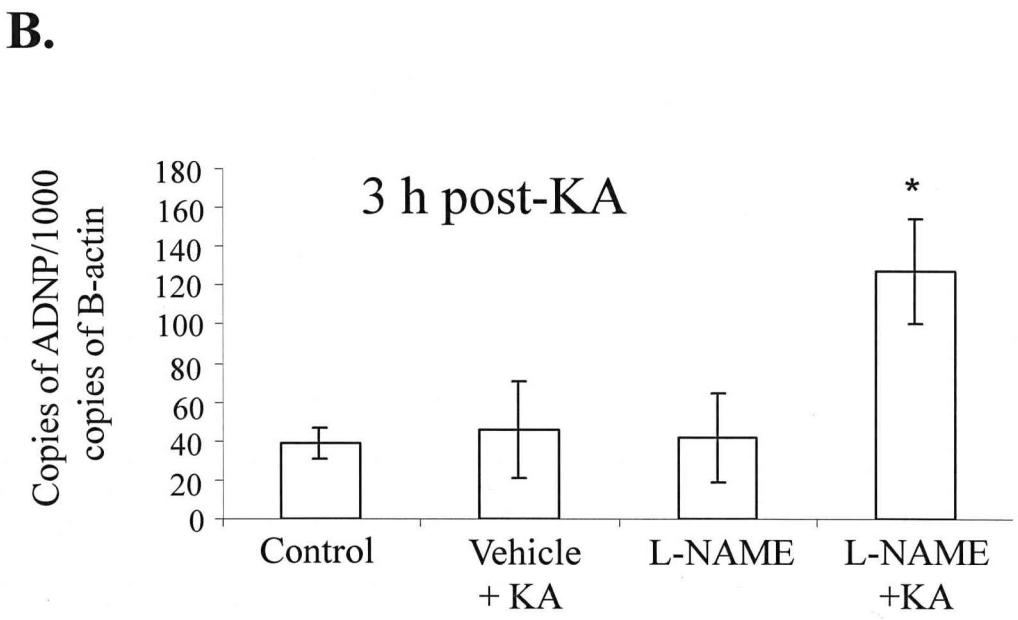


Figure 4.4 Effects of KA (3 hr post-KA) and L-NAME (3 hr) on ADNP mRNA and protein expression in the dentate gyrus. RT-PCR (A) and qPCR (B) analysis showed an increase in ADNP in L-NAME pre-treated animals compared with KA alone. L-NAME or KA on its own did not alter ADNP mRNA levels.

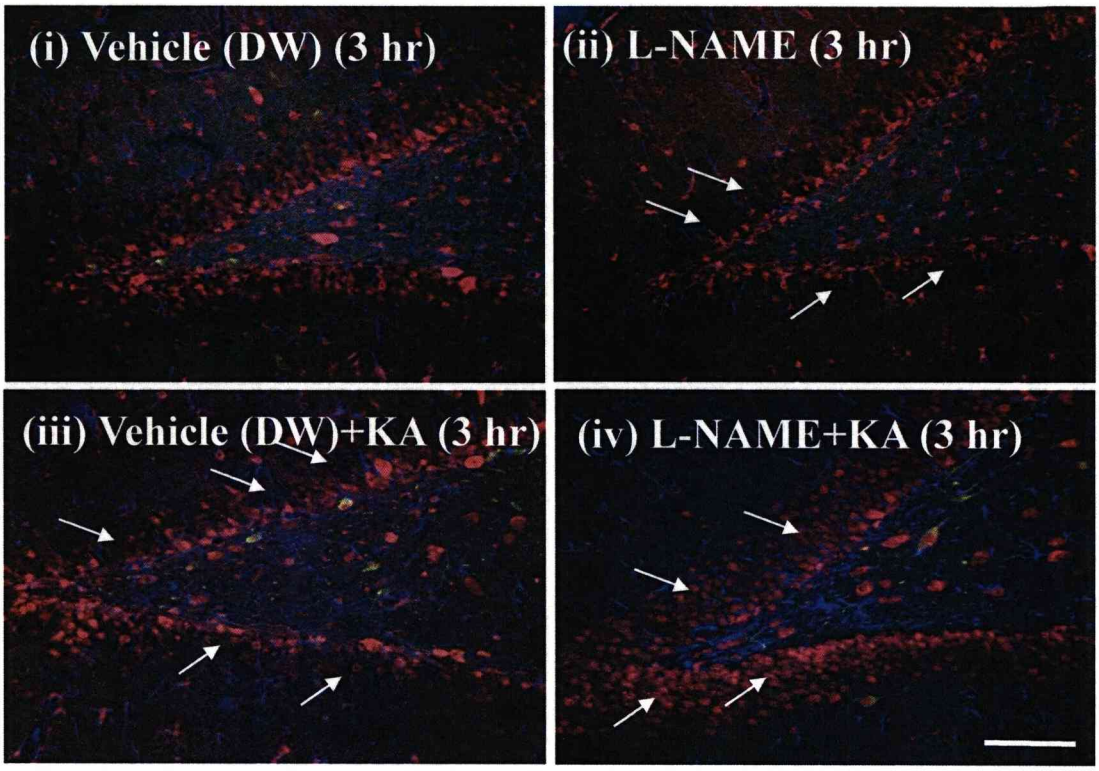


Figure 4.5 ADNP Immunostaining of L-NAME pre-treated animals confirmed an increase in ADNP$^+$ cells (red) in the SG and the hilus following KA-induced seizure (iv). L-NAME or KA on its own suppressed ADNP in many granule cells in the SG (indicated by arrows in (ii) and (iii) as examples, however, ADNP$^+$ hilar neurons varied. L-NAME (ii) suppressed ADNP in hilar neurons but KA (iii) did not. Green labelled cells in (i), (iii) and (iv) are nNOS$^+$ neurons which are absent in (ii). GFAP$^+$ cells (blue) appear to be down-regulated when treated with L-NAME (ii). Scale bar, 100 μm. D. Detailed analysis of ADNP + cell counts (neurons and astrocytes) from different layers of the dentate gyrus. KA on its own caused a slight decrease in ADNP+ cells in the OML and in the SG when compared with appropriate controls (** $p < 0.01$, n=3) but had no effect on the SGZ and the hilus. L-NAME treatment prior to KA significantly increased the number of ADNP+ granule cells in the SG, and marginally in the OML, compared with controls (*** $p< 0.001$, * $p< 0.05$, n=3). However, L-NAME on its own suppressed ADNP in a few granule cells in the SG compared to vehicle control (* $p< 0.05$).

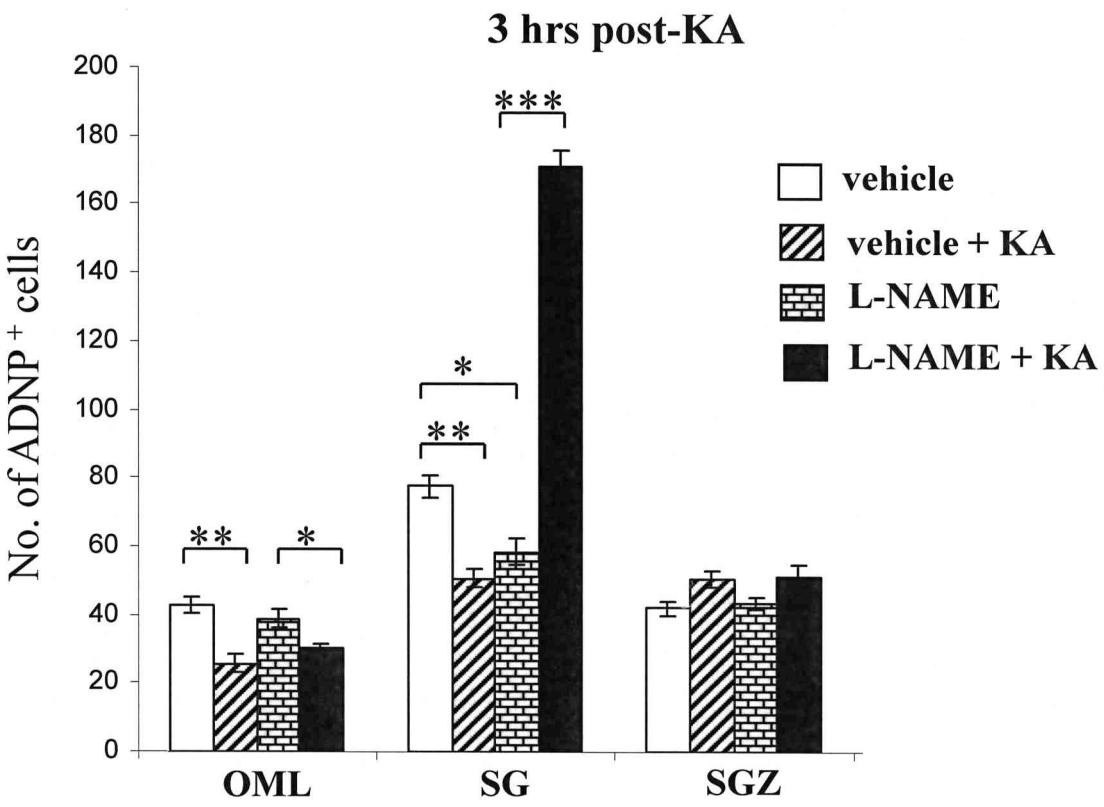


Figure 4.6 Detailed analysis of ADNP$^+$ cell counts (neurons and astrocytes) from different layers of the dentate gyrus. KA on its own caused a slight decrease in ADNP$^+$ cells in the OML and in the SG when compared with appropriate controls (** $p < 0.01$, n=3) but had no effect on the SGZ and the hilus. L-NAME treatment prior to KA significantly increased the number of ADNP+ granule cells in the SG, and marginally in the OML, compared with controls (*** $p< 0.001$, * $p< 0.05$, n=3). However, L-NAME on its own suppressed ADNP in a few granule cells in the SG compared to vehicle control (* $p< 0.05$).

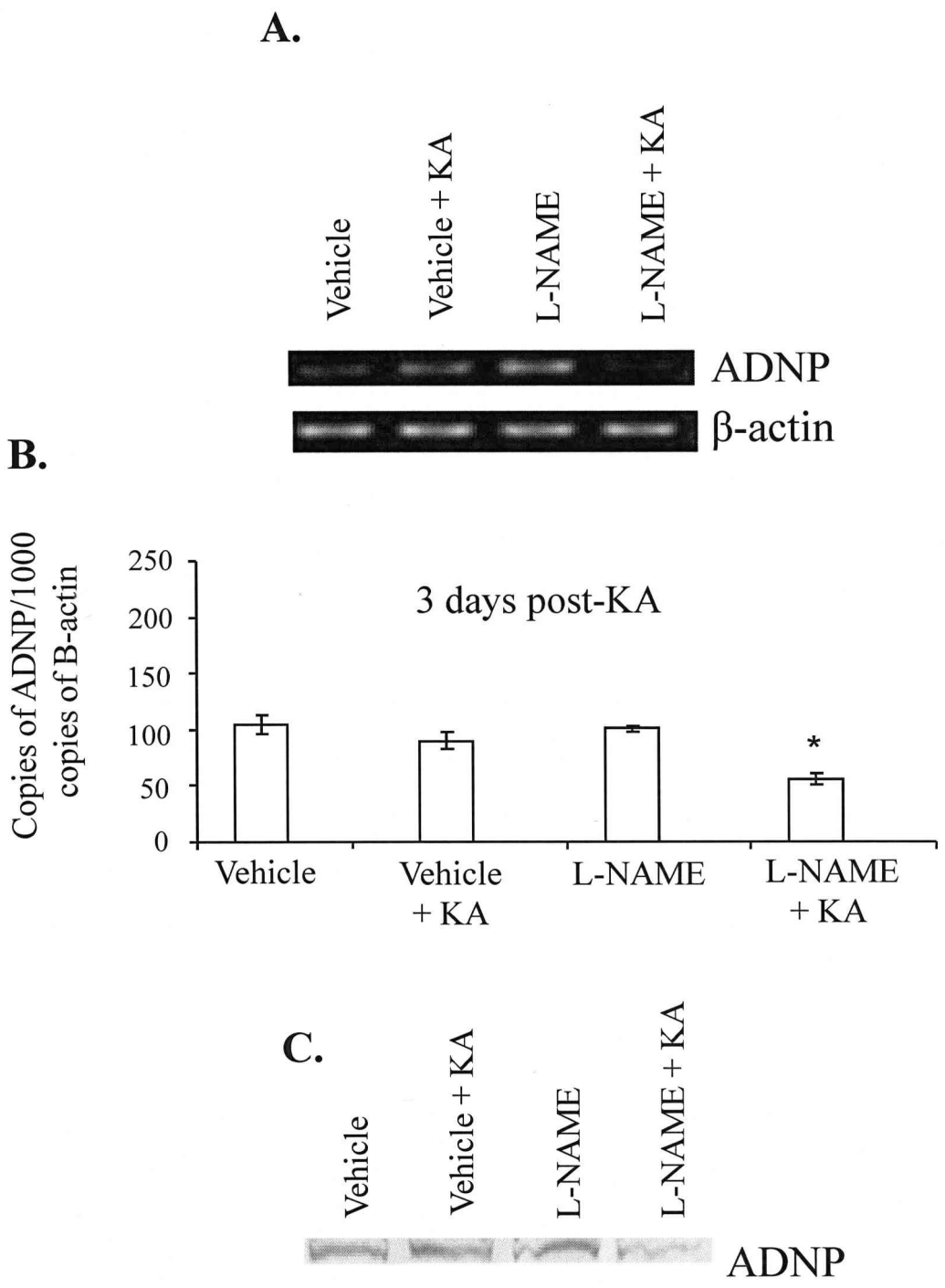


Figure 4.7 Effects of KA (3 d post-KA) and L-NAME (3 day group) on ADNP mRNA and protein expression in the dentate gyrus. RT-PCR (A) and qPCR (B) analysis showed a decrease of ADNP mRNA levels in L-NAME pre-treated animals compared with KA alone or other controls. L-NAME or KA on its own did not change ADNP mRNA levels. Western blot analysis (C) of proteins extracted from the dentate gyrus confirmed overall suppression of ADNP in the 3 d post-KA group that was pre-treated with L-NAME for > 24 hours.

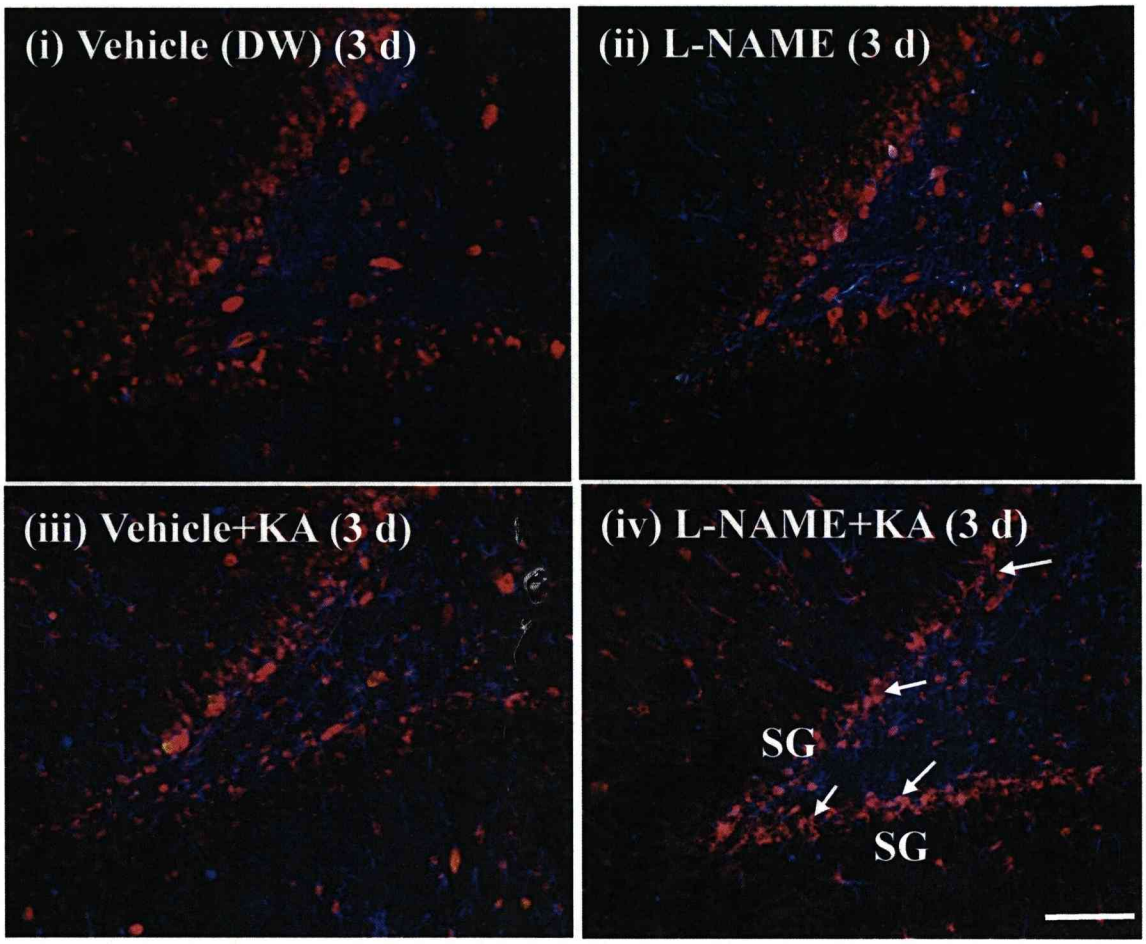


Figure 4.8 Immunostaining of L-NAME pre-treated animals (iv) confirmed a decrease of ADNP$^+$ cells (red) GFAP$^+$ (blue) in the SG and the hilus following KA-induced seizure. More cells in the SGZ were intensely stained for ADNP in L-NAME pre-treated (iv) animals (a few examples are indicated by arrows). KA (iii) or L-NAME (ii) on its own continue to suppress ADNP in many granule cells. L-NAME on its own appears to have a long-term effect on suppression of GFAP (ii). Scale bar, 100 μm.

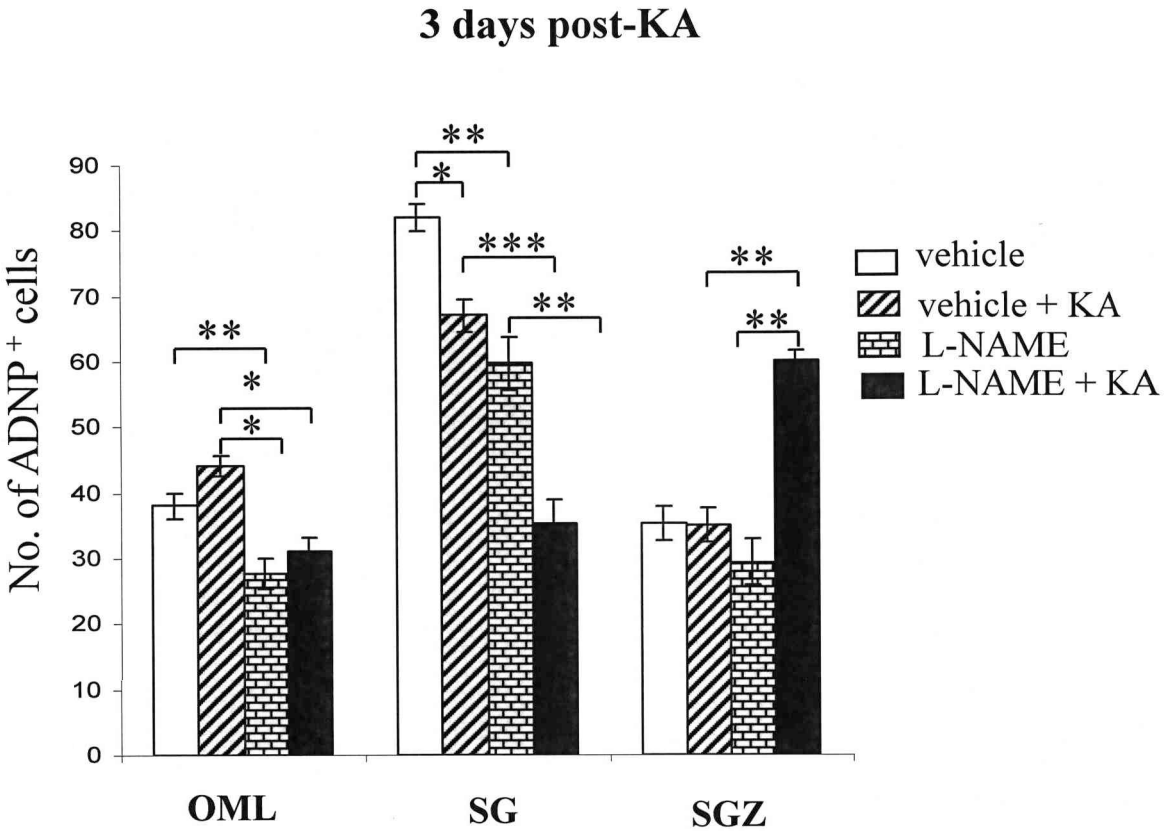
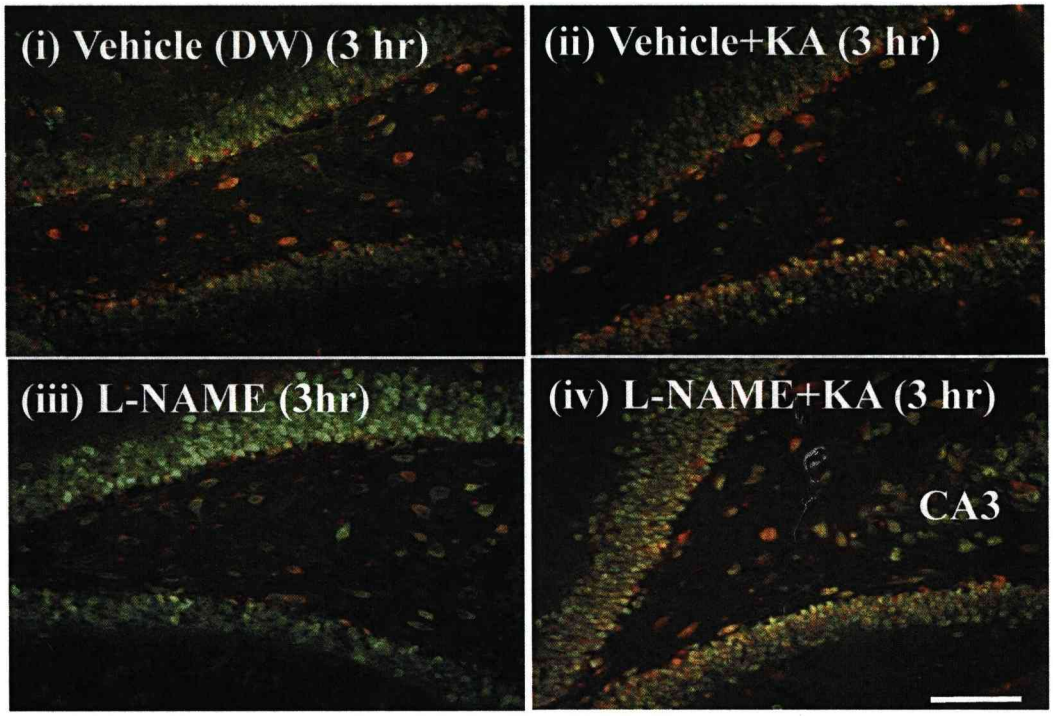


Figure 4.9 ADNP$^+$ cell counts (both neurons and astrocytes) from different layers of the dentate gyrus. KA alone suppressed ADNP in a few granule cells in the SG but had no effect on the OML, the SGZ and the hilus compared with appropriate controls. L-NAME prior to KA significantly increased the number of ADNP$^+$ granule cells in the SGZ compared with controls, but decreased in other layers except the hilus. However, L-NAME on its own decreases ADNP$^+$ cells in the OML and the SG compared to vehicle control (*$p< 0.05$, ** $p<0.01$, *** $p< 0.001$, $n=3$).


(i) Vehicle (DW) (3 hr)
(ii) Vehicle+KA (3 hr)
(iii) L-NAME (3hr)
(iv) L-NAME+KA (3 hr)
CA3

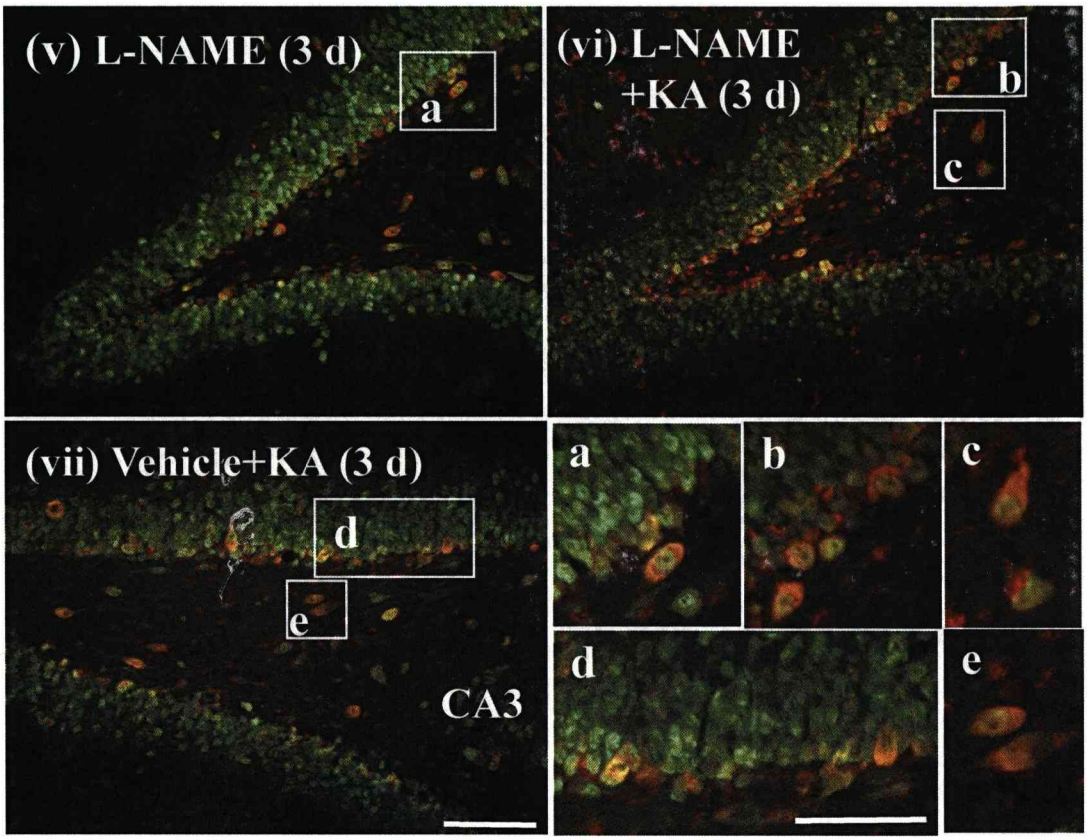


Figure 4.10 NeuN (green) and ADNP (red) immunostaining of the dentate gyrus from animals treated with L-NAME or vehicle with or without KA. The number of $NeuN^+$ hilar neurons containing ADNP was significantly reduced by 3 hr with L-NAME (iii) compared with the vehicle (i) and 3 days after the last dose of L-NAME (v) suggesting L-NAME mediated suppression of ADNP in some hilar neurons. By 3 hrs post-KA treatment (ii and iv) the average number of $ADNP^+$ hilar neurons did not change, but L-NAME pre-treatment caused a significant decrease in the number of $NeuN^+$ hilar neurons by 3 days post KA-injection (vi) and the vast majority of the remaining neurons in the hilus were $ADNP^+$. Higher magnification of the SGZ [(a), (b) and (d)] indicated by boxes in v) to vii) show a significant increase in the number of $ADNP^+$ cells in the SGZ (b). Morphology of the remaining $ADNP^+$ hilar neurons in the L-NAME pre-treated group (vi) indicated by a box (c) showed eccentric nuclei and irregular cell margins compared to the vehicle pre-treated group (vii) indicated by a box (e). Scale bar: 100 μm.

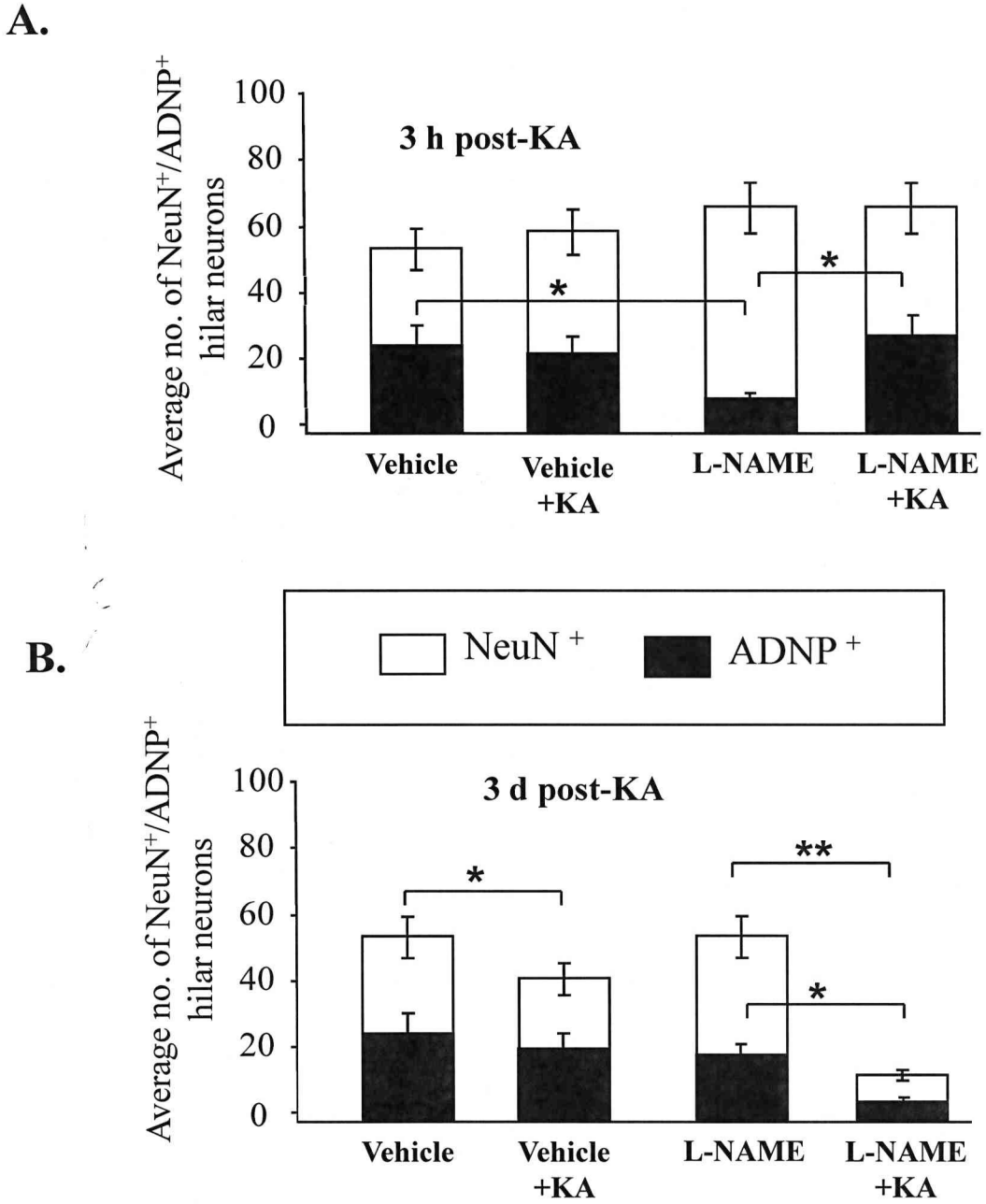


Figure 4.11 Hilar neuron counts from sections representing approximately the same region of the dentate gyrus from drug or vehicle treated animals. There was a significant reduction in $ADNP^+$ neurons in L-NAME treated (3 hr post-KA group; $p < 0.05$, n=3) compared to vehicle. KA had no effect on $ADNP^+$ at this time point. The number of $NeuN^+$ neurons also did not change between drug or vehicle treatment. However, in the 3 d post-KA group (B) KA caused a decrease in the number of $NeuN^+$ neurons compared with vehicle control ($p < 0.05$, ** $p< 0.01$, n=3) and $ADNP^+$ neurons also decreased in L-NAME pre-treated compared to vehicle pre-treated group $p < 0.05$, n=3).

4.4 Discussion

The DG is widely considered as an epileptogenic zone, however, other areas of the brain regions may be equally or even more important in orchestrating the generation and/or the control of spontaneous seizure (Heinemann et al., 1992; Bertram 1997, Bertram et al., 1998). The dentate gyrus is also an important region of the brain since it is the site of postnatal neurogenesis that occurs throughout the lifespan of the mammalian species. For a decade, there has been more emphasis on identifying the role of neurotrophins, such as BDNF in activity-dependent modification of synaptic plasticity in the hippocampus and the dentate gyrus (Tandon et al., 1999; Schinder and Poo, 2000; Simsek-Duran and Lonart, 2008). The previous chapter demonstrated the regulation of activity-dependent neuroprotective protein (ADNP) by the NO-cGMP pathway in the CA3 and CA1 regions of the hippocampus in the KA induced rat model of seizure (Cosgrave et al., 2008). In this chapter observations from the same animal model were extended to different layers of the DG.

It has been demonstrated in the DG of epileptic rats that kainate receptors contribute to an enhanced synchronised network activity (Epsztein et al., 2005). In the normal brain, the DG, the granule cells in particular, are less susceptible to cell death when compared to the CA3 and CA1 regions of the hippocampus. This is probably due to strong GABAergic inhibition and a high threshold for generation of action potential (Staley et al., 1992). Therefore, decrease in activity or loss of interneurons in the hilus and/or changes in the subgranular zone of the dentate gyrus may predispose this area of the brain to epileptogenesis.

In view of this, this chapter investigates the basal level expression of ADNP and its regulation by the NO-cGMP pathway in normal DG and in the KA-induced seizure model to understand the differential response of the layers of the dentate gyrus. L-NAME treatment alone caused suppression of ADNP in the hilar neurons in the 3 hr group, however, by three days ADNP expression returned to basal levels except in the KA-treated group implying hilar cell loss. Early morphological studies identified at least 21

types of interneuron in the dentate hilus alone (Amaral, 1978).It has been demonstrated that large numbers of hilar neurons die in the KA model of self sustained status epilepticus (SSSE) (Buckmaster & Jongen-Rêlo, 1999; Sloviter et al., 2003; Zappone and Sloviter, 2004). Amongst the hilar neurons, nearly 65% are mossy cells which are excitatory and the rest are believed to be inhibitory GABAergic interneurons. In the KA-SSSE model about 50% of mossy cells and 13% of GABAergic neurons were lost (Buckmaster & Jongen-Rêlo, 1999). Neither 7-NI nor ODQ caused any significant changes in ADNP expression in the DG implying that the neuronal source of NO and cGMP are not involved in ADNP regulation/expression in this region. It can be inferred, however, that NO may exert neuroprotective effects on interneurons via ADNP following seizure.

The seizure model used in this study is mild since the animals were treated with diazepam after the onset of the first generalised seizure. However, a recent study claims that a rat model similar to the one used in the present study is classified as a severe model (Yang et al., 2008). It has been previously demonstrated by Du et al. (1995) that diazepam rescues neurons from calcium-induced death in a KA model of epilepsy. But recent studies claim that diazepam's protective effect is limited to the CA region of the hippocampus (Pitkänen et al., 2005) which maybe why we see cell loss in the DG but not the hippocampus. Therefore, the decrease or absence of ADNP in a subpopulation of hilar neurons in the DG in KA-treated animals that were pre-treated with L-NAME suggests that the NO-ADNP pathway may modulate hilar neuronal survival following an initial seizure. This in turn could potentially contribute to additional recurrent excitatory circuitry, which subsequently may lead to epileptogenic events and SSSE if left for longer periods of time. Higher concentrations of NO are neurotoxic. Diazepam limits the severity of seizures (Pitkänen et al., 2005) and this would be predicted in turn to reduce excessive NO production by preventing excessive calcium influx, thus reducing the neurotoxic actions of NO (Rajasekaran, 2005; Chuang et al., 2007).

Large neurons in the hilus and neurons close to the SGZ did not show ADNP in their cell bodies following treatment with L-NAME but expression was seen again by 3 days.

These cells resemble basket cells or mossy cells in their morphology. It is speculative at this stage that basal levels of NO in the normal brain may cause ADNP production and expression in the granule cells of the SG. NO inhibition may facilitate the trafficking of ADNP towards dendrites, possibly, for modulating synaptic plasticity as increased ADNP expression was seen in nerve fibres in the outer molecular layer following L-NAME treatment by 3 hrs (Fig. 4.1 (iv)). It has been suggested that the neurotrophins undergo activity-dependent regulated release from neurons or glia, enabling them to act as synaptotrophins (Gall et al., 1989; Zafra 1990; Schinder et al., 2000). Exposure to neurotrophins also facilitates the induction of long-term potentiation (Figurov et al., 1996; Akaneya, 1997). NO may regulate ADNP for similar functions, possibly to modulate synaptic plasticity, neuronal survival and/or neurogenesis in the DG.

In summary, the DG of the normal brain contains $ADNP^{+}$ neurons and astrocytes which appear to be regulated by endogenous NO. Inhibiting NO by L-NAME treatment suppresses ADNP in the soma of granule cells and in hilar neurons. However, treating animals with L-NAME prior to KA-induced seizure causes ADNP synthesis in granule cells by 3 hrs which was later restricted to the SGZ by 3 days, but hilar neuron numbers were reduced and the remaining neurons showed eccentric nuclei and irregular cell boundaries. These results indicate that NO regulation of ADNP in the DG of normal and pathological brains may have different implications for epileptogenesis and that ADNP may be providing trophic support for new cells in the SGZ.

CHAPTER 5

The Regulation of VIP in the Hippocampus & Dentate Gyrus via the NO-cGMP Pathway Following Seizure

5.1 Introduction

Vasoactive Intestinal Peptide (VIP) was originally isolated from porcine duodenum and was described as having 'strong and unusually prolonged' vasodilator actions on the peripheral and splanchnic circulations (Said & Mutt, 1970). Since then, this neuropeptide has been found to be widely distributed in both the peripheral and central nervous systems (CNS) and is secreted by immune cells (Delgado et al., 2004). Its physiological effects have been described in the regulation of the pituitary gland, pancreas, adrenal glands, respiratory system, gastrointestinal tract, reproductive system and the immune system (Nussdorfer & Malendowicz, 1998; Bailey et al., 1990; Lundberg et al., 1984; Mazzocchi et al., 2002; Dalsgaard et al., 2003; Delgado et al., 2004). In the CNS, VIP can act as a neuromodulator, neurotransmitter, neurotrophic or neuroprotective factor (Gozes, 2002). It exerts its effect through specific membrane G protein-coupled receptors (GPCRs), where the C-terminal of the receptor protein is located in the extracellular space and the N-terminal is located inside the cell and can undergo glycosylation.

VIP is composed of 28 amino acids and belongs to a family of structurally related polypeptides including; pituitary adenylate cyclase activating polypeptide (PACAP), peptide histidine-isoleucine (PHI), peptide histidine-methionine (PHM), secretin, glucagon and glucagon-like peptide (GLP) and glucose-dependent insulinotropic peptide (GIP) (Sherwood et al., 2000). The main receptors characterized for VIP are PAC_1 (8 variants), which have a poor affinity for VIP and $VPAC_1$ and $VPAC_2$ receptors which have a much higher affinity for VIP. Upon binding to the latter two receptor sub-types, VIP causes activation of the adenylyl cyclase (AC) – cyclic adenosine 3'5'-mono-phosphate (cAMP) signal transduction pathway (Vaudry et al., 2000; Dejda et al., 2004). VIP receptors are the subject of current research interest for their role in circadian rhythm, cancer and neuroprotection (Vosko et al., 2007; Moody & Gozes, 2007; Brenneman 2007).

NO has been shown to regulate the release of VIP. In studies using nNOS knockout mice there was a decrease in the number of VIP immunopositive neurons and VIP mRNA in the cerebral cortex (Kim et al., 2003). In the suprachiasmatic nucleus (SCN), NO colocalizes with VIP in the subpopulation of nNOS-containing neurons, suggesting the involvement of NO, nNOS and VIP in circadian rhythm (Reuss et al., 1995). VIP is thought to play a role in neurodevelopment and has been linked to disorders such as autism, Downs Syndrome and foetal alcohol syndrome (FAS) (Hill 2007). VIP has also been implicated in epilepsy (de Lanerolle et al., 1995; Marksteiner et al., 1989). In a rodent model of epilepsy, VIP^+ neurons were spared in the hippocampus (Sloviter, 1987) and VIP receptor expression was shown to increase in human TLE (de Lanerolle et al., 1995). In the previous chapters it was found that ADNP, a VIP-responsive gene, is regulated by the NO-cGMP pathway in the hippocampus and via NO in the DG during basal conditions and post seizure. Following on from these observations, I have investigated the regulation of VIP following KA induced seizure and whether this is via an NO-cGMP dependent mechanism in the DG and hippocampus.

5.2 Materials and Methods

Materials and methods were carried out as previously described for in vivo injections of NO inhibitors and KA. Tissues were processed for immunofluorescence and RT-PCR, described in the methods section.

5.3 Results

5.3.1 Effect of NOS or sGC inhibition on VIP and GFAP expression in the dentate gyrus by 3 hours following KA

GFAP and VIP immunostaining revealed that VIP was predominantly present in astrocytes in the DG and that VIP was uniformly distributed in the cytoplasm of the cell bodies of astrocytes (Fig. 5.1A (v)). Astrocytic processes did not show VIP expression (Fig. 5.1A (v)). In the DG, VIP^+ cells were mainly distributed in the SGZ, the polymorphic cell layer and the hilus and were largely absent in the SG (Fig. 5.1A (i)).

Some $nNOS^+$ cells co-immunostained with VIP^+ cells, however, these were not quantified and this is the subject of further work.

To investigate whether the NO-cGMP pathway has a role in the expression of VIP following seizure, animals were treated with the NOS or sGC inhibitor with and without the induction of seizure by KA. VIP immunostaining did not show any significant differences in VIP immunopositive cells following treatment with the broad spectrum NOS inhibitor, L-NAME or the nNOS inhibitor 7-NI in the DG. However, the sGC inhibitor ODQ revealed a significant increase in VIP^+ cells compared to the appropriate control in the hilus, SGZ and SG (Fig. 5.1B, Fig. 5.2) by 3 hrs. However, following seizure (KA), ODQ treatment caused a significant decrease in the number of VIP^+ cells by 3 hours. A significant decrease in the number of $VIP^+/GFAP^+$ cells was also seen in this treatment group (Fig. 5.2). RT-PCR of the whole hippocampus using VIP primers revealed an increase in VIP mRNA expression by 3 hours (Fig. 5.9).

5.3.2 Effect of NOS or sGC inhibition on VIP and GFAP expression in the dentate gyrus by 3 days following KA

Immunostaining with VIP and GFAP showed that treatment with 7-NI and ODQ caused a significant increase in the number of VIP^+ cells (mainly in the SGZ) by 3 days compared to the appropriate control (Fig. 5.3B, Fig. 5.4). Following seizure, pre-treatment with the NOS inhibitor L-NAME caused a significant increase in the number of VIP^+ cells by 3 days (Fig. 5.3A, Fig. 5.4). RT-PCR of the whole hippocampus using VIP primers revealed no significant changes by 3 days in VIP mRNA expression (Fig. 5.9).

5.3.3 Effect of NOS or sGC inhibition on VIP and GFAP expression in the hippocampus by 3 hours following KA

VIP mRNA and protein was present in the DG and hippocampus as revealed by RT-PCR and immunostaining (Fig. 5.1, Fig. 5.5 & Fig. 5.9). Immunostaining revealed VIP expression in the CA3 was different to that with ADNP, as VIP staining was not seen in the pyramidal cell layer and was present mainly in astrocytes ($GFAP^+$ cells) in the CA1 and CA3 regions of the hippocampus (Fig. 5.5A, Fig. 5.5B, Fig. 5.7A & Fig. 5.7B). In

order to investigate whether the NO-cGMP pathway has a role in the expression of VIP in the hippocampus during basal conditions or following seizure, animals were treated with the NOS or sGC inhibitor as described previously.

Immunostaining with VIP showed that pre-treatment with L-NAME, 7-NI and ODQ caused no significant increase in the number of VIP^+ cells in the CA3 by 3 hours compared to the appropriate controls (Fig. 5.6). Co-immunostaining with GFAP also showed that no significant differences were observed in the number of $VIP^+/GFAP^+$ cells in any of the pre-treatment groups compared to the appropriate controls (Fig. 5.6). However, following seizure pre-treatment with L-NAME caused a significant decrease in the number of VIP^+ cells in the CA3 compared to the appropriate control by 3 hours (Fig.5.6). There was also a significant decrease in the number of $VIP^+/GFAP^+$ cells in the CA3 by 3 hours, compared to the appropriate control (Fig. 5.6).

5.3.4 Effect of NOS or sGC inhibition on VIP and GFAP expression in the hippocampus by 3 days following KA

Immunostaining with VIP also showed that pre-treatment with 7-NI and ODQ caused no significant increase in the number of VIP^+ cells in the CA3 by 3 days compared to the appropriate controls (Fig. 5.8).

Following seizure, pre-treatment with L-NAME caused a significant increase in the number of VIP^+ cells in the CA3 compared to the appropriate control (Fig.5.8). However, this increase in the number of VIP^+ cells was accompanied by a highly significant decrease in the number of $VIP^+/GFAP^+$ cells in the CA3 by 3 days, compared to the appropriate control (Fig. 5.8). RT-PCR of the whole hippocampus using VIP primers revealed no significant changes by 3 days in VIP mRNA expression (Fig. 5.9).

A.

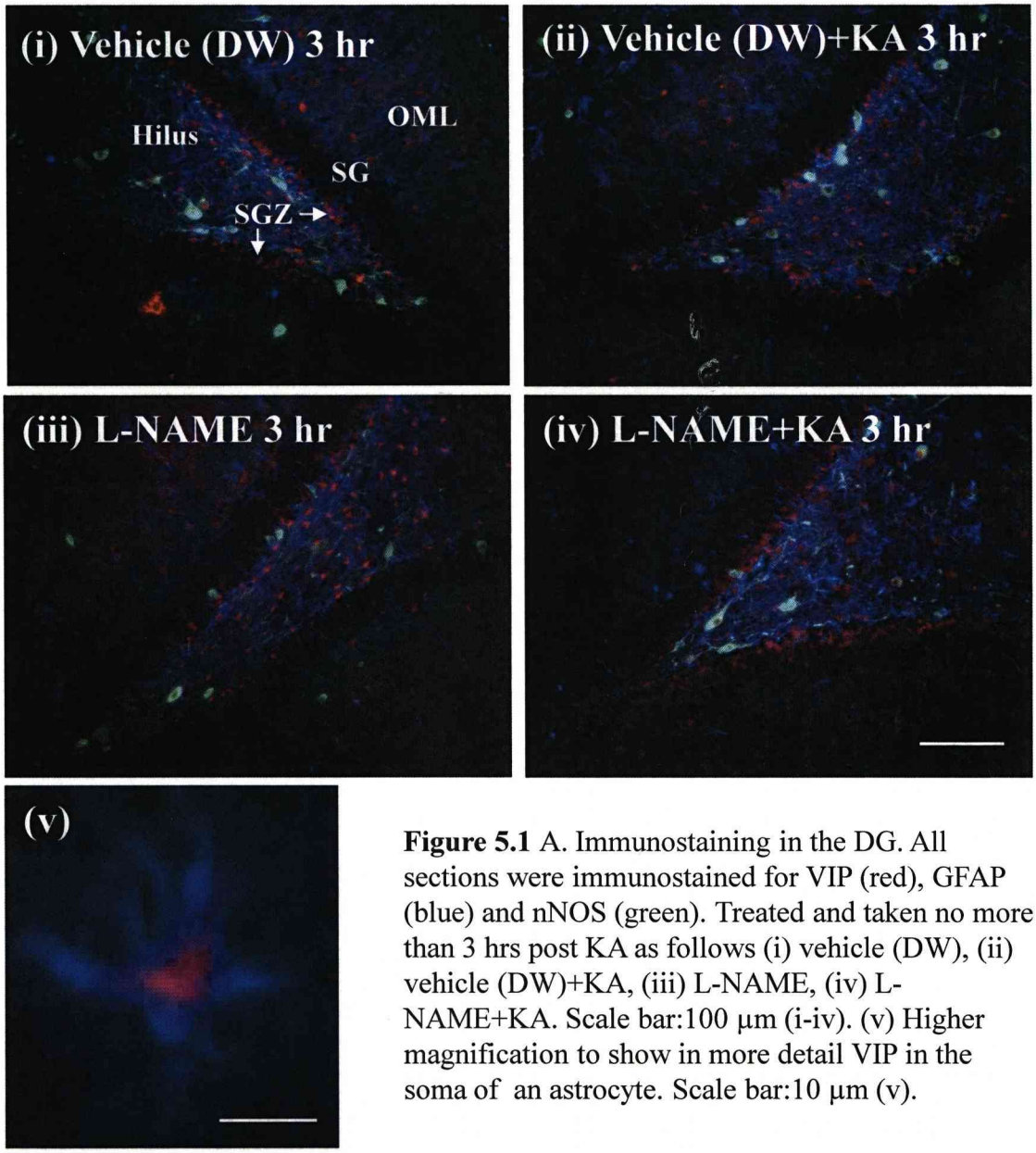


Figure 5.1 A. Immunostaining in the DG. All sections were immunostained for VIP (red), GFAP (blue) and nNOS (green). Treated and taken no more than 3 hrs post KA as follows (i) vehicle (DW), (ii) vehicle (DW)+KA, (iii) L-NAME, (iv) L-NAME+KA. Scale bar:100 μm (i-iv). (v) Higher magnification to show in more detail VIP in the soma of an astrocyte. Scale bar:10 μm (v).

B.

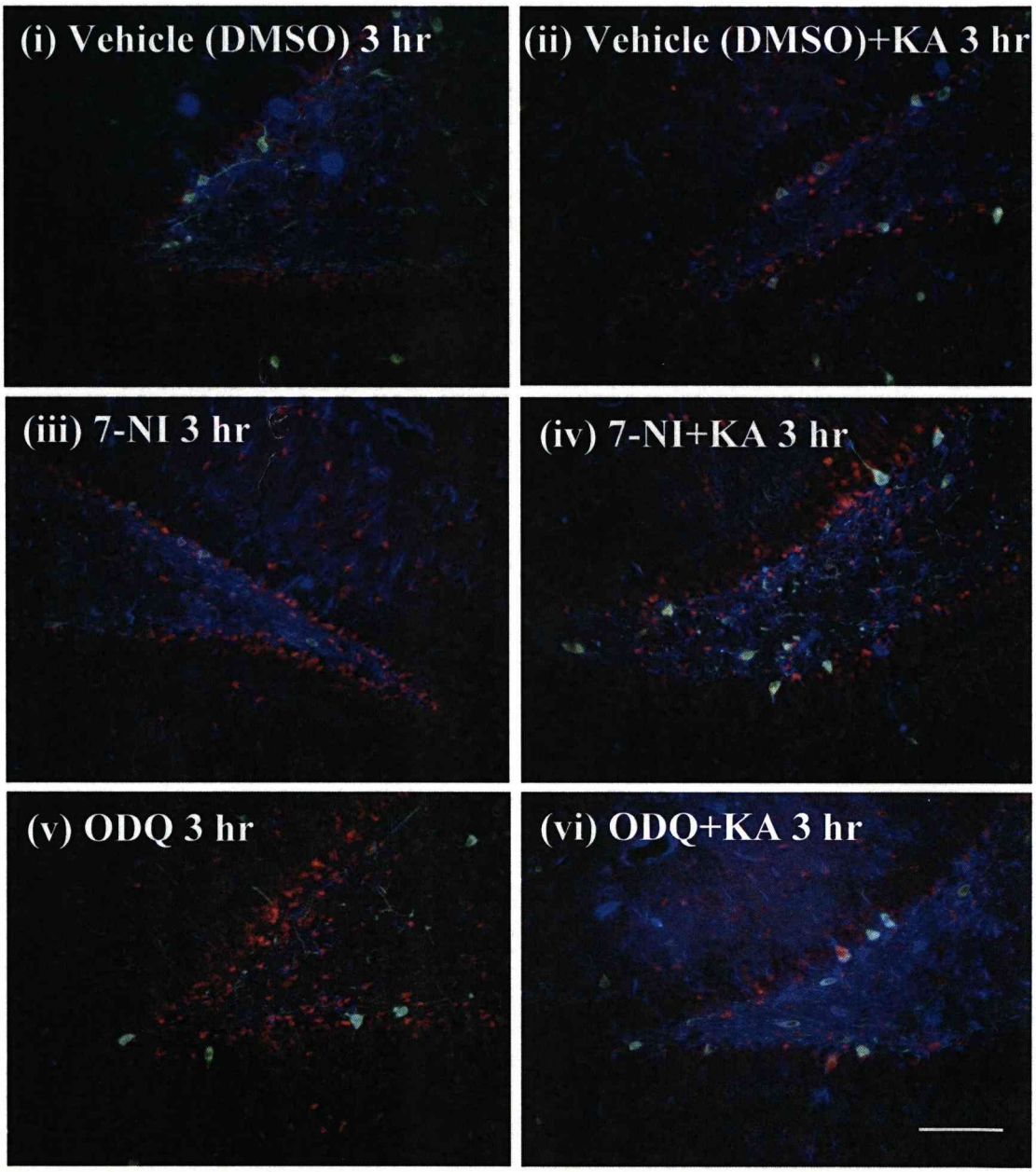


Figure 5.1 B. Immunostaining in the DG. All sections were immunostained for VIP (red), GFAP (blue) and nNOS (green). Treated and taken no more than 3 hours post KA as follows (i) vehicle (DMSO), (ii) vehicle (DMSO)+KA, (iii) 7-NI, (iv) 7-NI+KA, (v) ODQ, (vi) ODQ+KA. ODQ treatment caused an increase in VIP staining in the hilus, SGZ and SG. Scale bar:100 μm

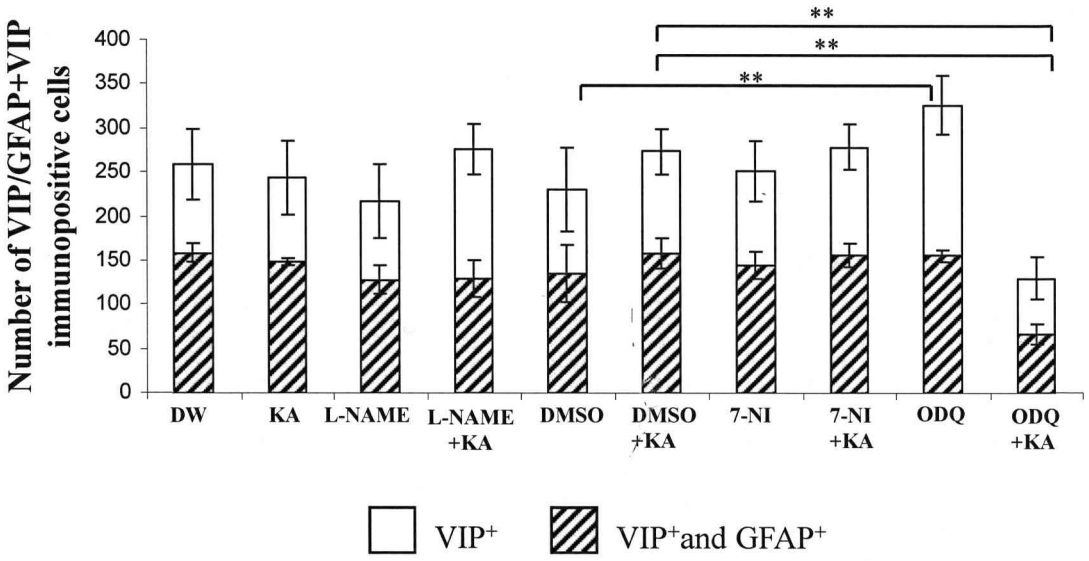


Figure 5.2 Quantification of VIP+ and VIP+/GFAP+ cells in the DG at 3 hours for treatment with; vehicle (DW), vehicle (DW)+KA, L-NAME, L-NAME+KA, vehicle (DMSO), vehicle (DMSO)+KA, 7-NI, 7-NI+KA, ODQ, ODQ+KA. Following sGC inhibition with ODQ an increase in the number of VIP^+ cells was observed, conversely a decrease in the number of VIP+ and GFAP+/VIP+ cells was observed with ODQ and KA treatment (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$; $n = 3$).

A.

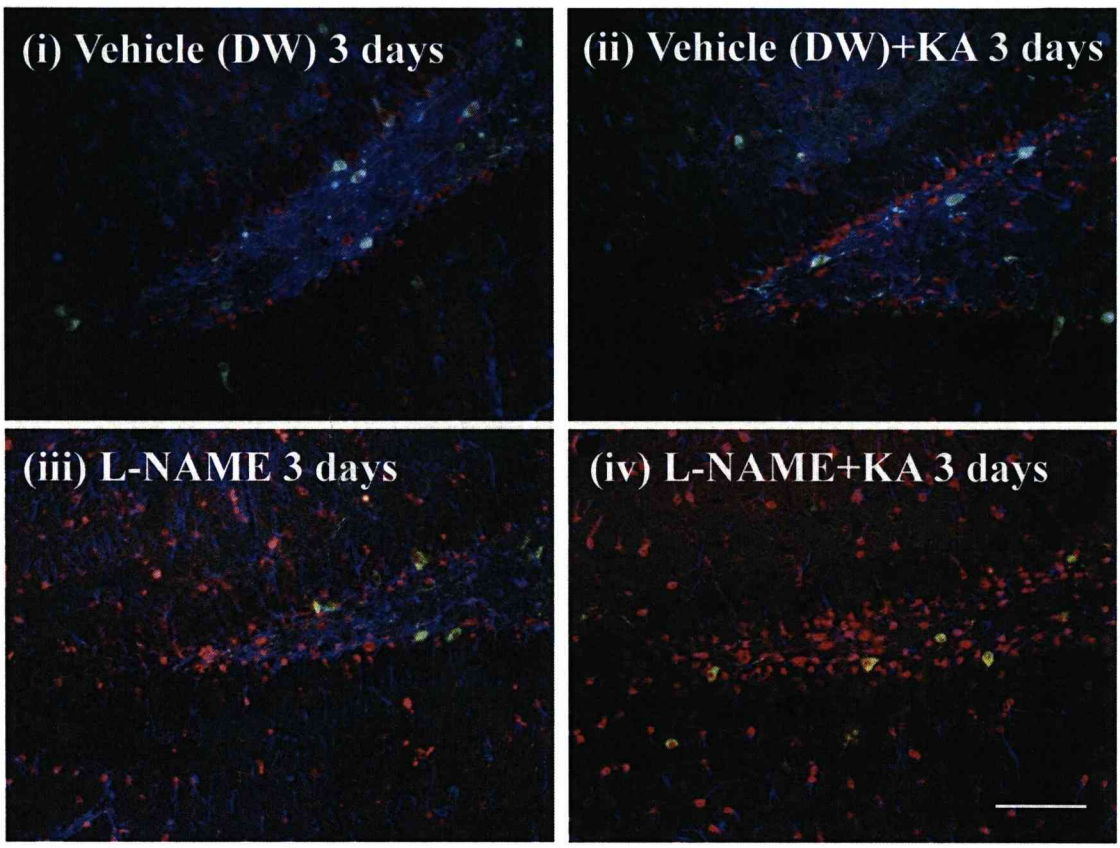


Figure 5.3 A. Immunohistochemistry in the DG at 3 days. All sections were immunostained for VIP (red), GFAP (blue) and nNOS (green). 3 days post KA NO inhibition caused an increase in the number VIP^+ cells on the OML, SGZ and hilus. Scale bar:100 μm

B.

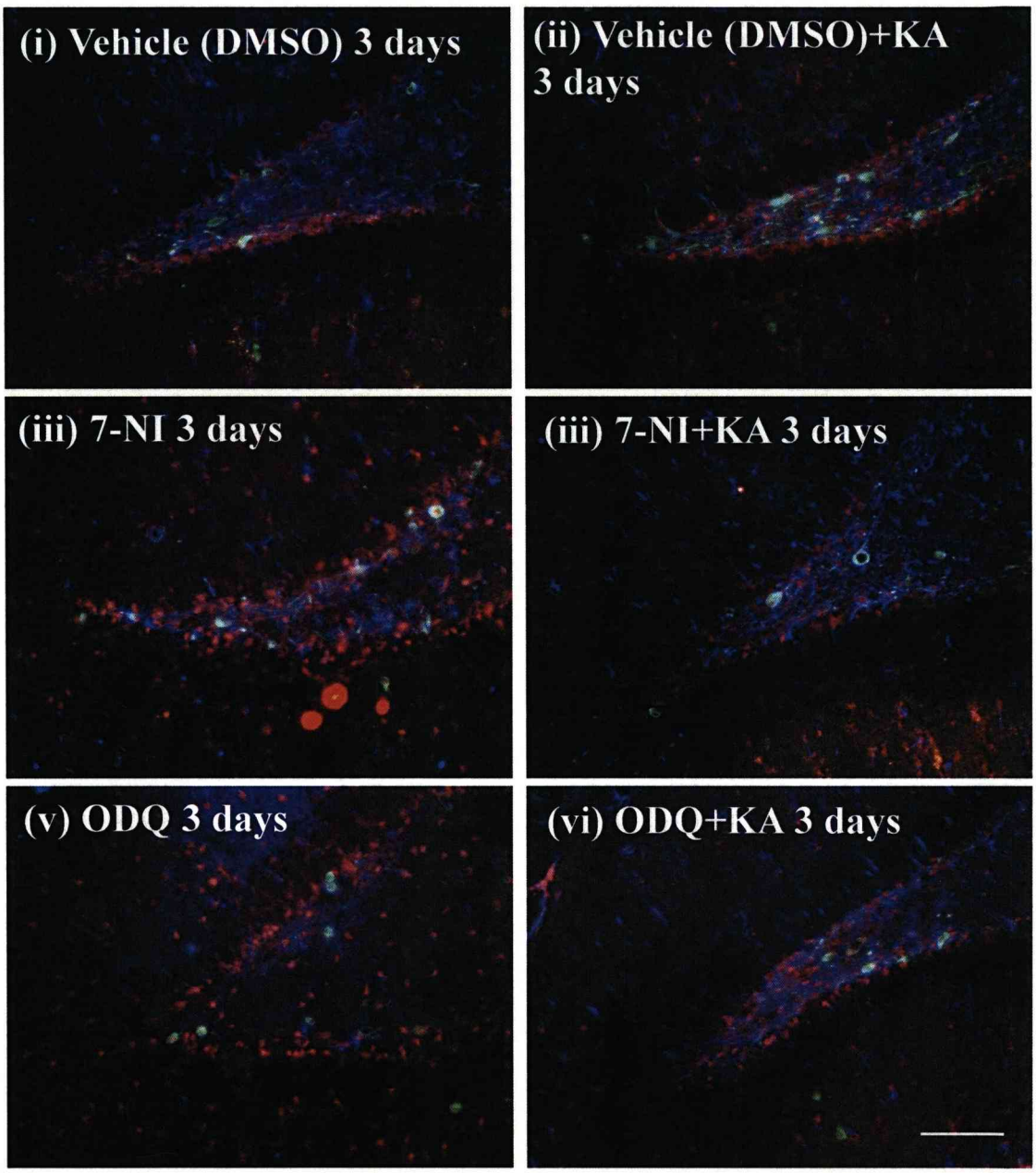


Figure 5.3 B. Immunohistochemistry in the DG. All sections were immunostained for VIP (red), GFAP (blue) and nNOS (green). Treated and taken 3 days post KA as follows (i) vehicle (DMSO), (ii) vehicle (DMSO)+KA, (iii) 7-NI, (iv) 7-NI+KA, (v) ODQ, (vi) ODQ+KA. Scale bar:100 μm

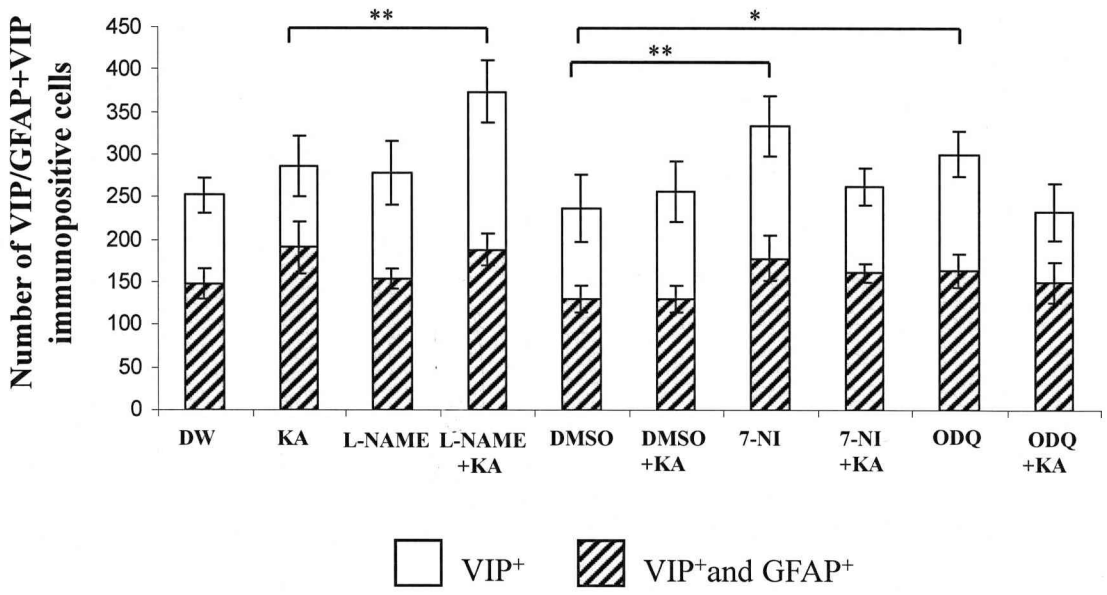


Figure 5.4 Quantification of VIP+ and VIP+/GFAP+ cells in the DG at 3 days for treatment with; vehicle (DW), vehicle (DW)+KA, L-NAME, L-NAME+KA, vehicle (DMSO), vehicle (DMSO)+KA, 7-NI, 7-NI+KA, ODQ, ODQ+KA. A significant increase in the number of VIP^+ cells was seen with 7-NI and ODQ pre-treatment and L-NAME+KA caused an increase in the number of VIP^+ cells in the DG also, suggesting the role of NO in VIP and seizure (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$; $n = 3$).

A.

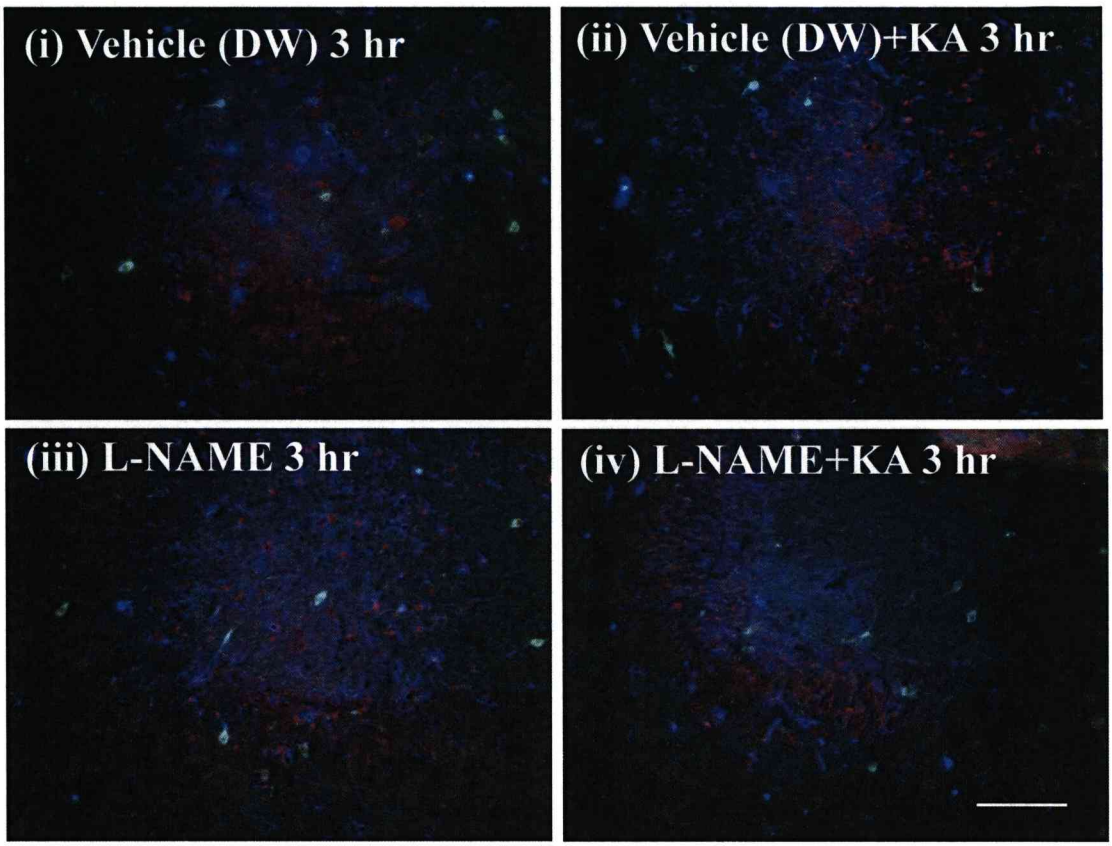


Figure 5.5 A. Immunostaining in the CA3. All sections were immunostained for VIP (red), GFAP (blue) and nNOS (green). Treated and taken no more than 3 hours post KA as follows (i) vehicle (DW), (ii) vehicle (DW)+KA, (iii) L-NAME, (iv) L-NAME+KA. NO inhibition caused a decrease in VIP+ cells by 3 hrs following seizure (L-NAME+KA treatment) Scale bar:100 μm

B.

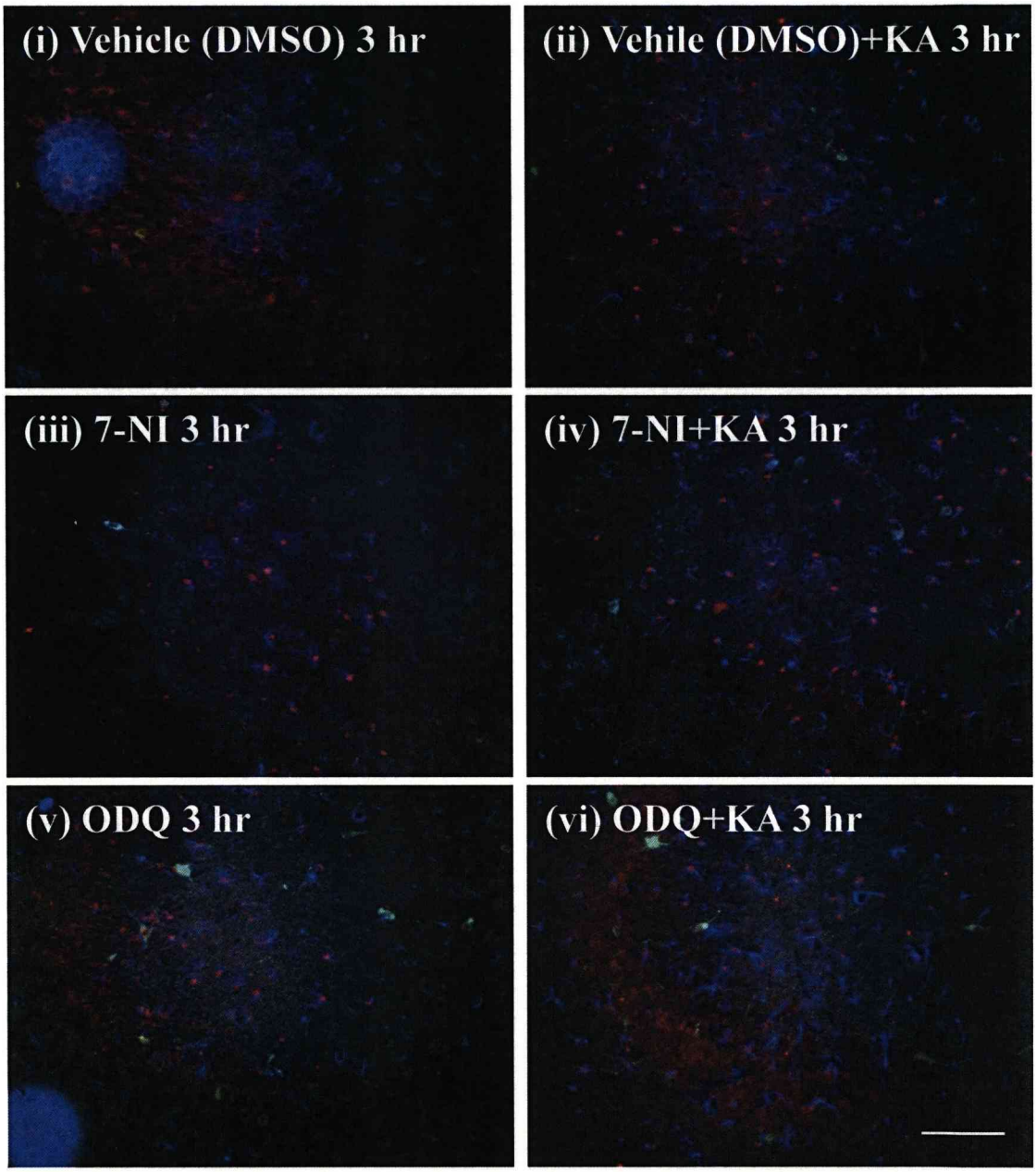


Figure 5.5 B. Immunohistochemistry in the CA3. All sections were immunostained for VIP (red), GFAP (blue) and nNOS (green). Treated and taken no more than 3 hours post KA as follows (i) vehicle (DMSO), (ii) vehicle (DMSO)+KA, (iii) 7-NI, (iv) 7-NI+KA, (v) ODQ, (vi) ODQ+KA. No significant changes were observed with any of the NOS/ sGC inhibitors. Scale bar:100 μm

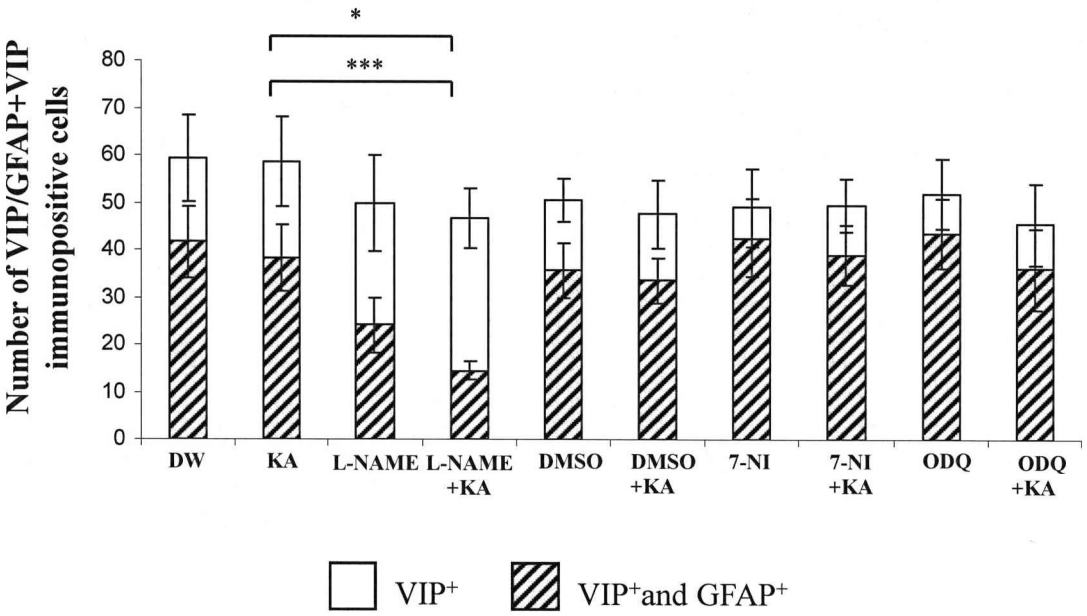


Figure 5.6 Quantification of VIP+ and VIP+/GFAP+ cells in the CA3 at 3 hours for treatment with; vehicle (DW), vehicle (DW)+KA, L-NAME, L-NAME+KA, vehicle (DMSO), vehicle (DMSO)+KA, 7-NI, 7-NI+KA, ODQ, ODQ+KA. Following seizure, L-NAME pre-treatment caused a significant decrease in the number of VIP^+ cells in the CA3. (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$; $n = 3$).

A.

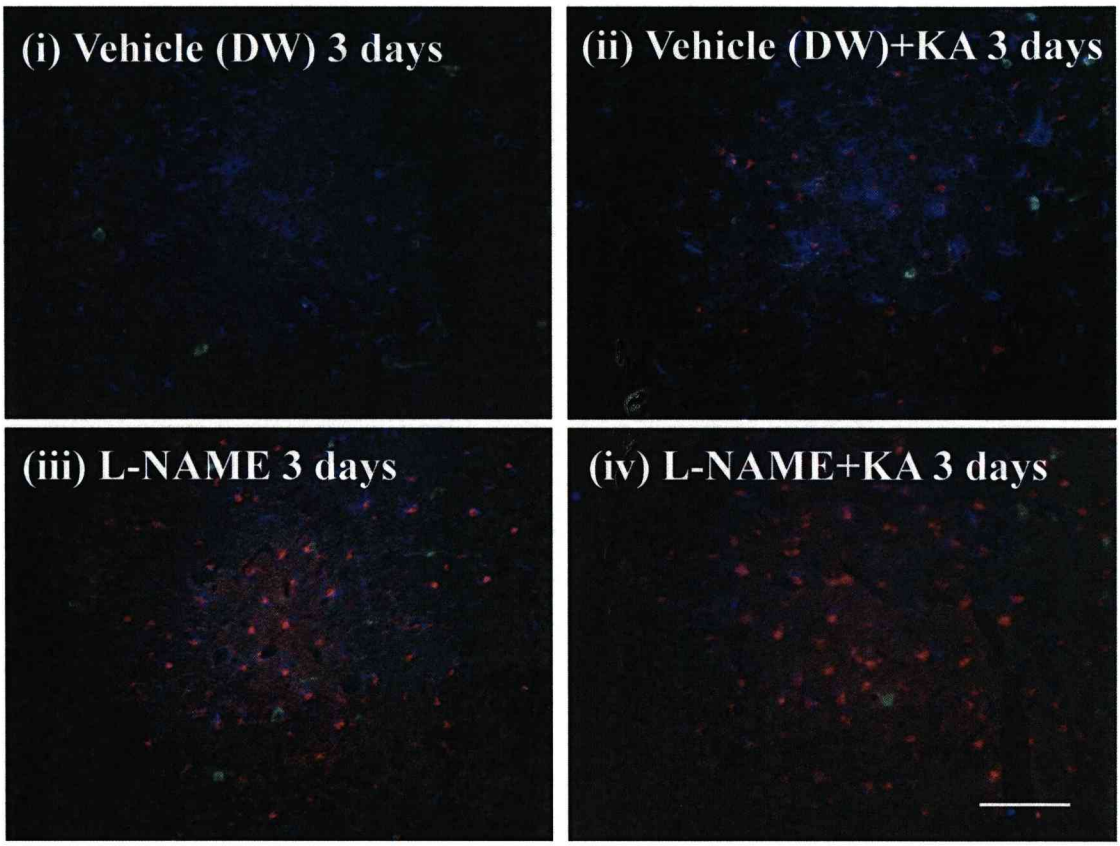


Figure 5.7 A. Immunohistochemistry in the CA3 at 3 days. All sections were immunostained for VIP (red), GFAP (blue) and nNOS (green). Treated and taken 3 days post KA as follows (i) vehicle (DW), (ii) vehicle (DW)+KA, (iii) L-NAME, (iv) L-NAME+KA. By 3 days NO inhibition caused an increase in the number of VIP^+ cells post-KA. Scale bar:100 μm

B.

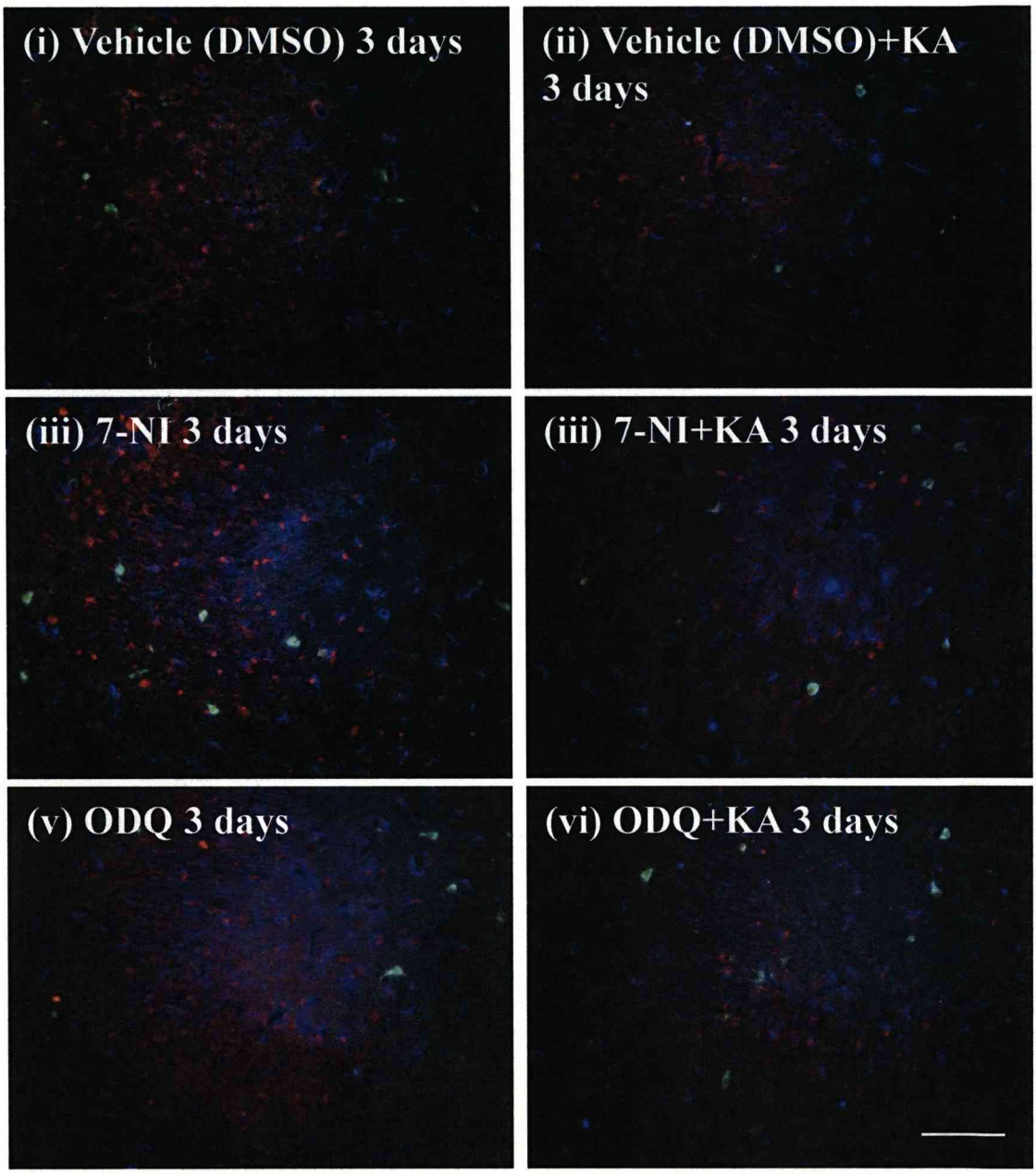


Figure 5.7 B. Immunohistochemistry of the CA3. All sections were immunostained for VIP (red), GFAP (blue) and nNOS (green). Treated and taken 3 days post KA as follows (i) vehicle (DMSO), (ii) vehicle (DMSO)+KA, (iii) 7-NI, (iv) 7-NI+KA, (v) ODQ, (vi) ODQ+KA. Scale bar:100 μm

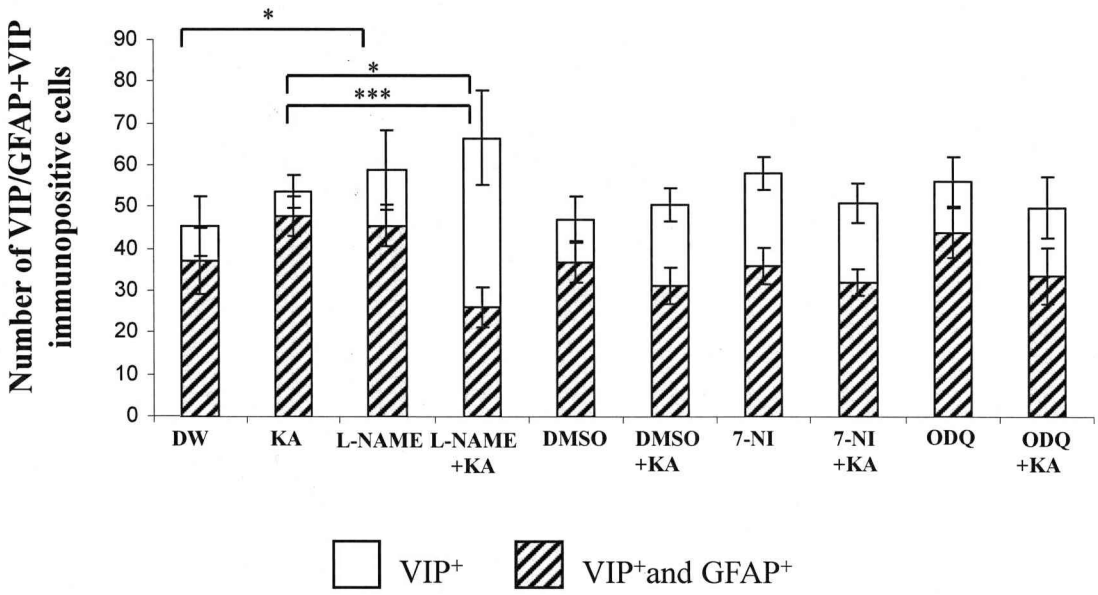


Figure 5.8 Quantification of VIP+ and VIP+/GFAP+ cells in the CA3 at 3 days for treatment with; vehicle (DW), vehicle (DW)+KA, L-NAME, L-NAME+KA, vehicle (DMSO), vehicle (DMSO)+KA, 7-NI, 7-NI+KA, ODQ, ODQ+KA. Cell counts revealed an increase in the the number of VIP^+ cells corresponded with a decrease in the number of $GFAP^+/VIP^+$ cells (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$; $n = 3$).

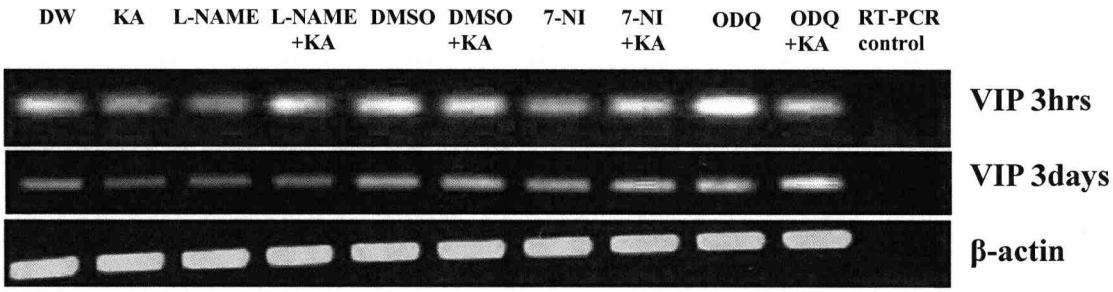


Figure 5.9 RT-PCR analysis of VIP mRNA revealed that ODQ pre-treatment causes an increase in VIP mRNA by 3 hours. Treating with the sGC inibitor ODQ prior to KA causes a decrease in VIP mRNA by 3 hours.

5.4 Discussion

The role of VIP has previously been described in both *in vitro* and *in vivo* models with various neurotoxic insults such as ethanol, hydrogen peroxide (H_2O_2) and β-amyloid. Here, the regulation of VIP via the NO-cGMP pathway *in vivo* following KA-induced seizure was investigated. Following a seizure, pathological changes take place as described previously (see introduction, section 1.6), these events could serve to change the hippocampal circuitry to potentiate the next seizure or could be to protect cells from subsequent cell death. The neuroprotective mechanism of action of VIP is still largely unknown but it is thought to be via activation of the adenylyl cyclase (AC)-cAMP→ protein kinase A (PKA) and mitogen –activated protein (MAP) kinase pathways and inhibition of caspase 3 (Nowak et al., 2007). It has also been suggested that VIP may exert its neuroprotective effects via a mechanism involving astrocytes and that these VIP-stimulated astrocytes secrete neuroprotective/neurotrophic proteins such as activity-dependent neurotrophic factor (ADNF), activity-dependent neuroprotective protein (ADNP), interleukin (IL)-1 and 6, macrophage inflammatory protein (MIP), neurotrophin-3, protease nexin-1 and RANTES (Brenneman et al., 2003; Dejda et al., 2005; Zusev & Gozes, 2004). Conversely, VIP can inhibit the production of inflammatory mediators in activated microglia which in turn, may lead to neurodegenerative processes. These results showed that the sGC inhibitor, ODQ caused a significant increase in VIP^+ cells by 3 hours in the DG (Fig. 5.2), suggesting that the NO-cGMP pathway is involved in the regulation of VIP at basal levels in the DG. However, following a seizure, ODQ caused a significant decrease in the number of VIP^+ cells by 3 hours in the DG. A significant decrease in the number of $VIP^+/GFAP^+$ cells was also seen in this treatment group (Fig. 5.2), suggesting the involvement of the NO-cGMP pathway in the DG in the regulation of VIP and glial activation following seizure.

VIP-mediated cyclic AMP responses have been shown to be larger in glial cultures compared to neuronal cultures. This suggests that astrocytes may be the primary target for VIP (Nowak et al., 2007). It has also been shown in the rat brain, that pathological conditions such as hypoxia can lead to changes in VIP-driven cyclic AMP-dependent signalling, this may also be the case for the NO-cGMP pathway where NO may be

influencing molecular processes occurring at the level of receptor protein or receptor-Gs protein coupling. In the previous chapters immunostaining has shown ADNP expression in both neurons and glia. It may be that ADNP secreted from glia acts on neurons. These results show that VIP expression is mainly in astrocytes (Fig. 5.1A (v)). Following a seizure, this VIP glial regulation could indicate a mechanism to increase the excitability of neurons via ADNP following the initial seizure, configuring the circuitry for subsequent seizures or it could be activating the processes involved in cell survival and the potential for injury in a seizure focus.

An autopsy study of hippocampi taken from patients with TLE with extrahippocampal temporal lobe lesions revealed a significant increase in VIP receptors in the dentate molecular layer and lowers levels in the CA fields and subiculum compared to normal patients. However, in patients with no temporal lobe lesions but considerable hippocampal neuronal loss there was a significant increase in VIP receptor binding in all CA fields and subiculum (de Lanerolle et al., 1995). Although the findings in this chapter showed no significant changes after a seizure, they did, however, reveal changes in VIP expression following NO inhibition, providing an insight into the mechanism by which VIP is regulated in the initial stages following a seizure.

These results showed that following a seizure, NO inhibition caused a significant decrease in the number of VIP^+ cells in the CA3 compared to the appropriate control by 3 hours (Fig.5.6), there was also a significant decrease in the number of $VIP^+/GFAP^+$ cells in the CA3 by 3 hours, compared to the appropriate control (Fig. 5.6). This suggests that NO is involved in the regulation of VIP and glial activation following a seizure. NO is well known for its role in neural transmission (Garthwaite 2008) and its role in neuron-glia communication is also becoming increasingly evident (Thippeswamy et al., 2007) and it is clearly established that astrocytes are involved in synaptic transmission and synaptogenesis. Factors released by glial cells that participate in neural communication are termed 'gliotransmitters' (Volterra & Meldolesi, 2005). Endozepines, the endogenous ligands for benzodiazepine receptors can act as gliotransmitters. These are derived from diazepam-binding inhibitor (DBI), an 86-amino acid polypeptide that is exclusively

expressed in astrocytes (Guidotti et al., 1983; Bürgí et al., 1999). VIP can stimulate the release of endozepines via the PAC_1 receptor (Masmoudi et al., 2003). Consequently, VIP-induced endozepine release may also indirectly regulate GABAergic transmission (Papadopoulos et al., 2006) and so during seizure VIP could indirectly be acting as the brain's endogenous diazepam via an NO mediated mechanism. Alternatively, this could present a mechanism via which patients with refractory epilepsy do not respond to anti-epileptic drugs.

In summary, in the DG, following seizure, VIP expression appears to be mediated via an NO-cGMP mechanism since the inhibition of sGC decreases VIP expression. However, in the hippocampus, VIP appears to be regulated by NO following seizure. This may be via a glial-mediated mechanism.

CHAPTER 6

The Effects of Nitric Oxide on ADNP and Cell Proliferation in the Dentate Gyrus Following Seizure

6.1 Introduction

Neurogenesis involves the proliferation, survival, differentiation and maturation of dividing precursor cells. The DG is one of the few regions of the adult brain where this phenomenon takes place. New granule cells are generated from dividing precursor cells located in the SGZ, the border between the hilus and the granule cell layer (Fig. 6.1). In the rat dentate gyrus, it is estimated that approximately 9000 new cells are formed every day (Cameron & McKay, 2001). Within the first few days after mitosis, the fate of these precursor cells is determined and the factors deciding whether they die or differentiate into mature neurons are not yet fully understood (Dayer et al., 2003). New neurons integrate into the pre-exisiting hippocampal circuitry and by four weeks their electrophysiological properties are identical to those of mature neurons (van Praag et al., 2002). This integration of new neurons into the hippocampal circuitry is thought to be important for adapting to novel situations, e.g. enriched environments increase neurogenesis and promote learning and memory (Kempermann, 2002; Gould et al., 1999). However, stress and pathological conditions can also have an effect on neurogenesis.

Seizures can alter the amount and pattern of neurogenesis depending on the type of seizure. Acute seizures are thought to augment neurogenesis and the migration of newly born neurons into ectopic regions such as the hilus and molecular layer. Animal models of TLE have shown prolonged seizures lead to an increase in the proliferation of granule cell progenitors in the DG (Parent et al., 1997; Bengzon et al., 1997; Gray et al., 1998). It is postulated that this aberrant migration and integration of new neurons may contribute to an epileptogenic hippocampal circuitry following acute seizures, status epilepticus and traumatic brain injury (Parent et al., 1997). However, reduced neurogenesis is associated with recurrent spontaneous seizures e.g. with chronic temporal lobe epilepsy in humans (Hattiangady et al., 2004).

NO has been implicated in epilepsy and is thought to have a pathophysiological role in convulsions. Studies on patients with intellectual disability (ID) who suffer from epilepsy

showed higher levels of NO metabolites and IL-6 in serum compared to ID patients without epilepsy (Carmeli et al., 2008). NO is also believed to negatively regulate neurogenesis (Pacer et al., 2003). It is becoming increasingly evident that ADNP plays a role in development, cancer cell growth and neurogenesis. The human ADNP gene was mapped to the chromosome 20q12-13.2, a region that is amplified in many neuroplasias and is associated with cancer cell growth (Bassan et al., 1999; Zamostiano et al., 2001; Gozes, 2007). ADNP knockout mice exhibit neural tube closure defects and death at E8.5-9.5 (Pinhasov et al., 2003). Also ADNP-deficient embryos exhibit a decrease in organogenesis/neurogenesis-related transcripts. Heterozygous ADNP+/- mice display neuronal/glial pathology and reduced cognitive functions (Viluh-Shultzman et al., 2007). In chapter 4, immunohistochemistry and PCR revealed that NO inhibition caused an increase in ADNP expression in the subgranular zone of the dentate gyrus by 3 days following seizure. The role of ADNP in hippocampal neurogenesis has not been previously investigated. The aim of this chapter was to investigate whether these effects seen in the dentate gyrus in chapter 4 were associated with neurogenesis.

6.2 Materials and Methods

Materials and methods were carried out as previously described for in vivo injections of NO inhibitors and KA. Tissues and cultures were processed for BrdU immunostaining as described in the methods section.

6.3 Results

6.3.1 In vivo effect of NO inhibition on ADNP and BrdU expression in the dentate gyrus following KA

Four groups of animals were treated with BrdU (100mg/kg) prior to administration of vehicle (DW), vehicle (DW)+KA, L-NAME, L-NAME+KA. BrdU and ADNP immunostaining revealed that BrdU staining was predominantly present in the SGZ of the DG and that ADNP was uniformly distributed in this cytoplasm of cells in this area (Fig. 6.1A). Following KA treatment there were no significant changes in $BrDU^+$ cells compared to vehicle control (Fig. 6.1A(v), Fig. 6.1A(ii)Fig. 6.2). However, following L-NAME treatment there was a significant increase in the number of $BrDU^+$ cells (Fig.

6.1A(viii), Fig. 6.2) compared to vehicle control (Fig. 6.1A(ii)Fig. 6.2). Notably, this increase in the number of $BrDU^+$ cells appeared to be in ectopic regions such as the hilus and molecular layer, indicated by white arrows (Fig. 6.1A(viii)).

Following seizure, NO inhibition caused a significant increase in the number of $BrDU^+$ cells in the SGZ (Fig. 6.1A(xi), Fig. 6.2), which was accompanied by a significant increase in the number of $BrDU^+$ and $ADNP^+$ cells (Fig. 6.1A(xii), Fig. 6.2).

Immunostaining with doublecortin (DCX) (a cell marker for newly born neurons) of the DG from animals treated with L-NAME+KA, showed that some of these $BrdU^+$ cells were also immunopositive for DCX (Fig. 6.3A(i),(ii)) suggesting a relationship between NO ADNP and neurogenesis.

6.3.2 In vitro expression of ADNP in DG cells and the effect of NO inhibition following KA treatment

To investigate these effects further, endogenous precursor cells were isolated from the rat DG and grown in vitro. Hence, the hippocampi of 7-10 day old wistar rats were removed and the DG dissected from the rest of the hippocampus (Fig. 2.2). DG cells were plated and grown in vitro (Fig. 6.4A) for 7 days before treatment. Immunostaining with TUJ-1, NeuN showed the differentiation of cells into neurons (Fig. 6.4A(iv), Fig. 6.5A).

Triple immunostaining with TUJ-1, ADNP and BrdU, showed that both neuronal and proliferating cells expressed ADNP (Fig. 6.5B). Triple immunostaining with DCX, ADNP and BrdU revealed that ADNP was also expressed in newly born neurons (Fig. 6.6A), however, staining with GFAP, BrdU and ADNP only showed ADNP to be present in some but not all astrocytes (Fig. 6.6B).

Double immunostaining with ADNP and BrdU revealed that NO inhibition caused a significant increase in the number of $BrDU^+$ cells (Fig. 6.7A(xi), Fig. 6.8) compared to KA alone (Fig. 6.7A(v), Fig. 6.8), which was accompanied by a significant increase in the number of $BrDU^+$ and $ADNP^+$ cells (Fig. 6.7A(xi), Fig. 6.8) compared to KA alone (Fig. 6.7A(v), Fig. 6.8).

Cells grown in vitro at 9 DIV for long pulse BrDU and 7 DIV for short pulse BrDU were treated with vehicle (DW), vehicle (DW)+KA, L-NAME, L-NAME+KA and immunostained for BrdU, NeuN, ADNP, GFAP and nestin. Merged images showed no

co-localisation with BrdU and NeuN (Fig. 6.9A (i)), however, an increase in $ADNP^{+}$ astrocytes was seen with NO inhibition is shown following KA (Fig. 6.9A (iii)) compared to control (Fig. 6.6B (iv)). Immunostaining for nestin, a precursor cell marker, showed co-localisation of BrdU and nestin (Fig. 6.9A(iv)) suggesting that NO and ADNP may be involved in the proliferation of precursor cells following seizure.

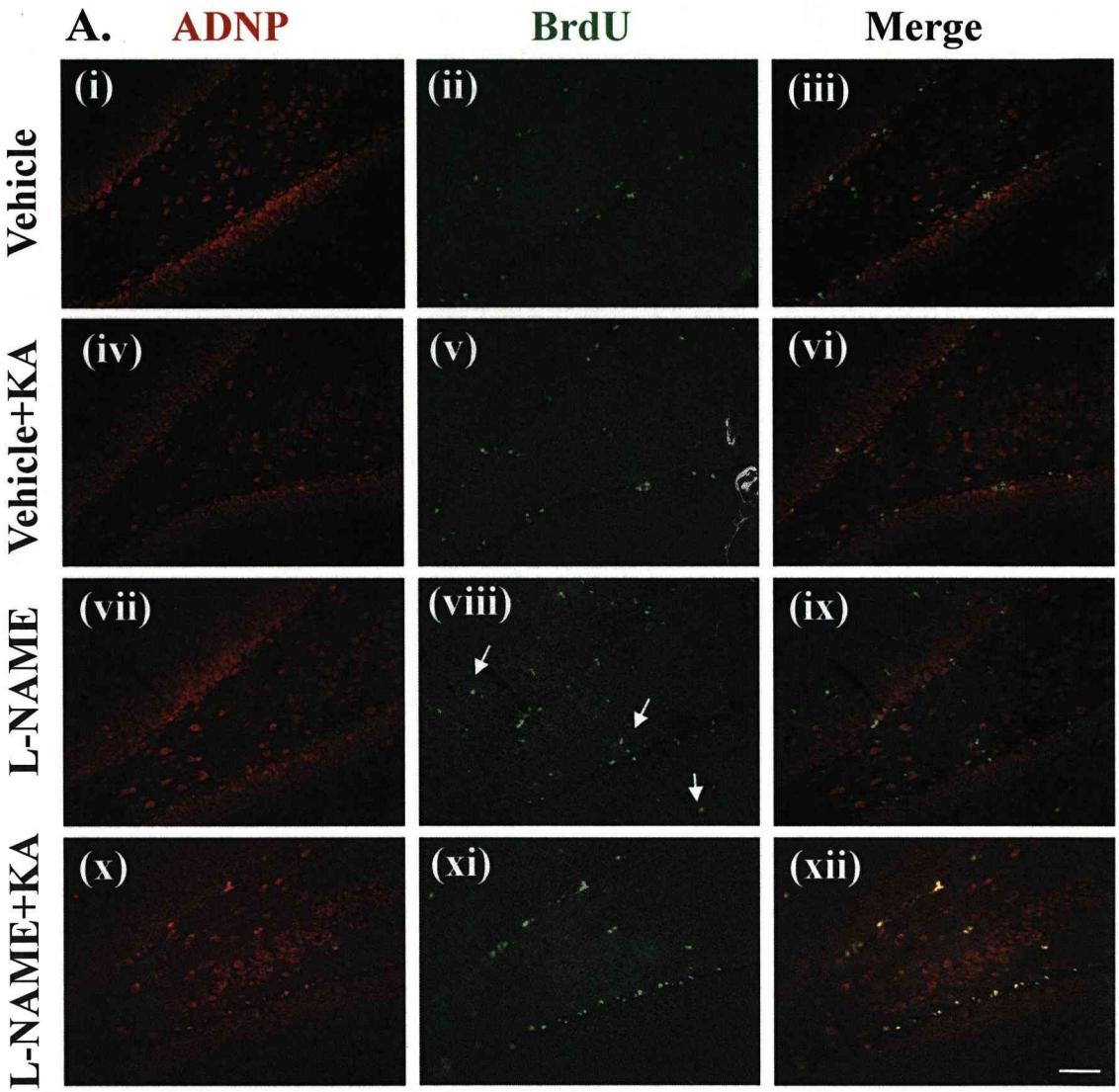


Figure 6.1 Photomicrographs of the DG taken from animals treated with BrdU prior to administration of vehicle (DW), vehicle (DW)+KA, L-NAME, L-NAME+KA. Immunostaining with ADNP (red) and BrdU (green) shows increased hippocampal neurogenesis with NO inhibition following seizure (xi). (viii) shows an increase in ectopic neurogenesis (indicated by white arrows). Scale bar: 100μm.

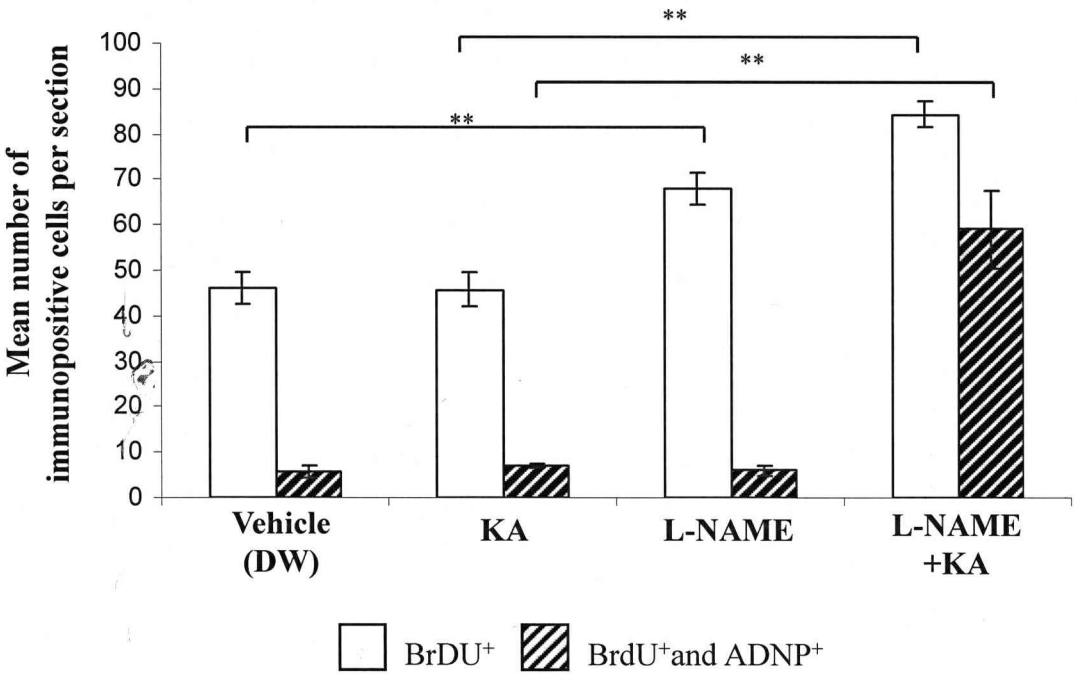


Figure 6.2 Histogram of cell counts to show increased hippocampal neurogenesis with NO inhibition and NO inhibition following seizure. Values are expressed as mean ± SEM. Significant differences are determined by one-way ANOVA with Bonferroni post-hoc test (*p <0.05, **p <0.01, ***p <0.001).

A.

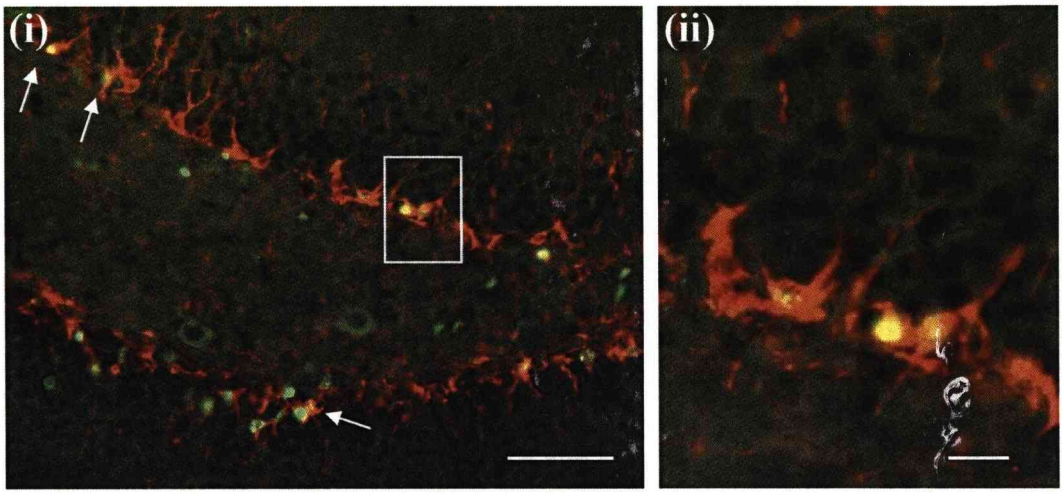


Figure 6.3 Photomicrographs of the DG taken from an animal treated with BrdU prior to administration of L-NAME+KA.(i) Immunostaining with doublecortin (DCX) (red) and BrdU (green) shows $BrdU^+$ cells co-localise with doublecortin, a cell marker for newly born neurons (indicated by arrows). Immunostaining with ADNP (red) and BrdU (green) revealed an increase in neurogenesis in the SGZ following seizure with L-NAME pre-treatment. Scale bars: 100μm in (i) and 20μm in (ii).

A.

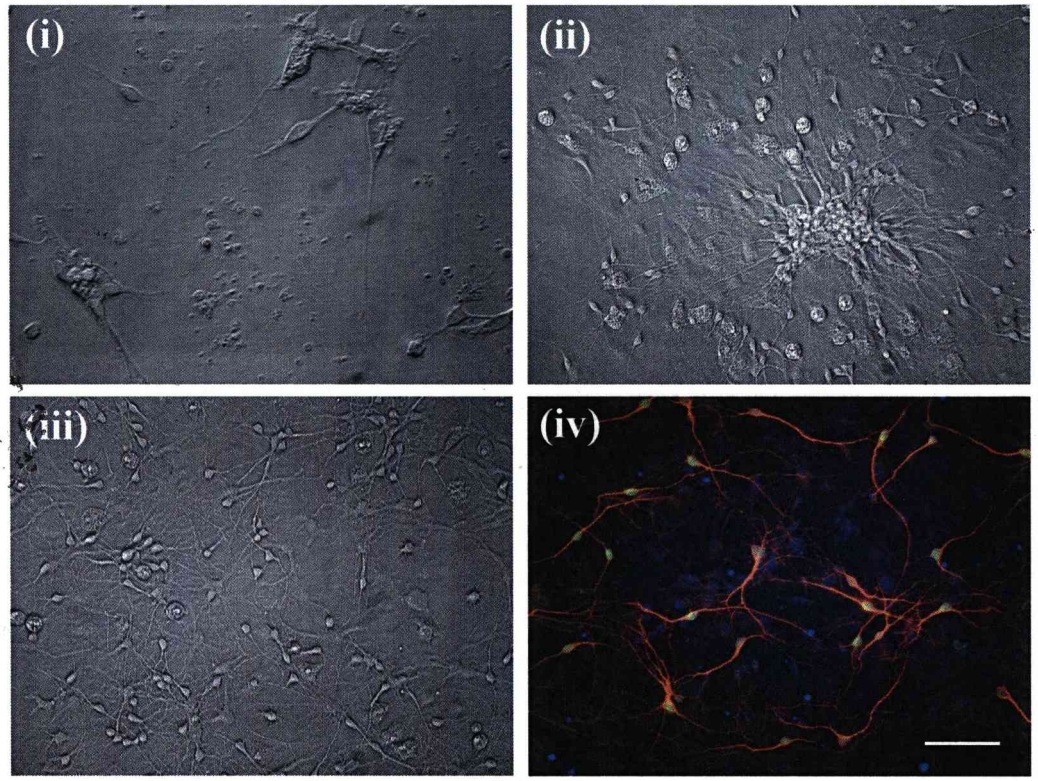


Figure 6.4 Photomicropgraphs of DG cells grown in vitro at (i) 2 days in vitro (DIV), (ii) 4 DIV, (iii) 6 DIV showing the proliferation and migration of cells in vitro. (iv) Merged image of immunostaining with TUJ-1 (red), NeuN (green) and ADNP (blue) to show differentiation of neuronal cells in vitro. Scale bar: 100μm.

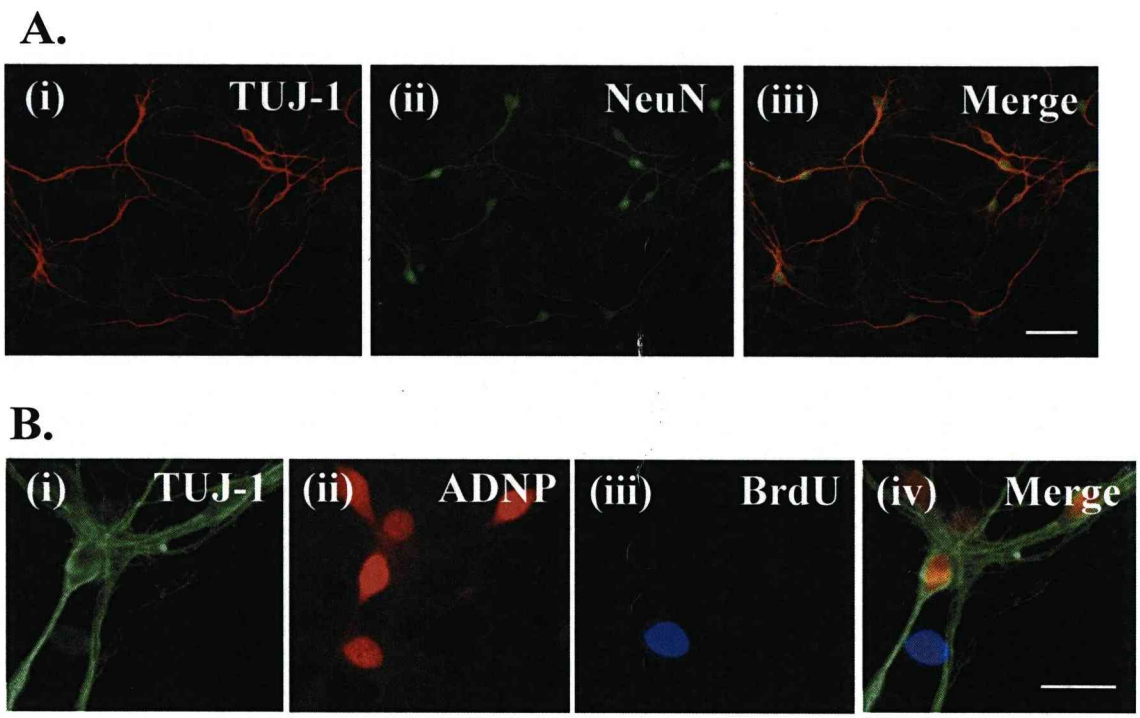


Figure 6.5 Photomicropgraphs of DG cells grown in vitro at (i) 7 DIV, treated with BrdU and immunostained in (A) with Tuj-1 (red), NeuN (green) and in (B) Tuj-1 (green), ADNP (red) and BrdU (blue). (iv) Merged image of immunostaining with TUJ-1 (green), ADNP (red) and BrdU (blue) show ADNP is expressed in the cytoplasm of both neurons and proliferating cells. Scale bar: 50μm in (A) and 20μm in (B).

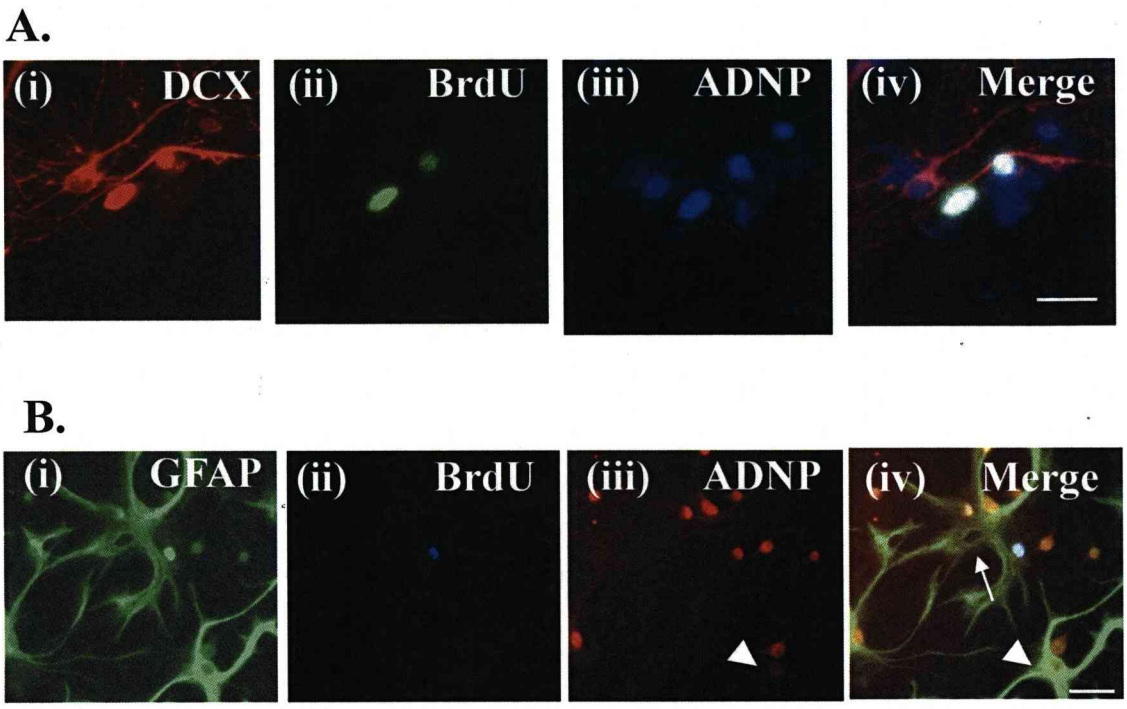


Figure 6.6 Photomicropgraphs of DG cells grown in vitro at (i) 7 DIV, treated with BrdU and immunostained in (A) with DCX (red), BrdU(green), ADNP (blue) and in (B) GFAP (green), BrdU (blue) and ADNP (red). Merged images of immunostaining with ADNP show ADNP is expressed in the cytoplasm of both newly born neurons and is some (indicated by an arrowhead), but not all, astrocytes (indicated with an arrow). Scale bars: 20μm

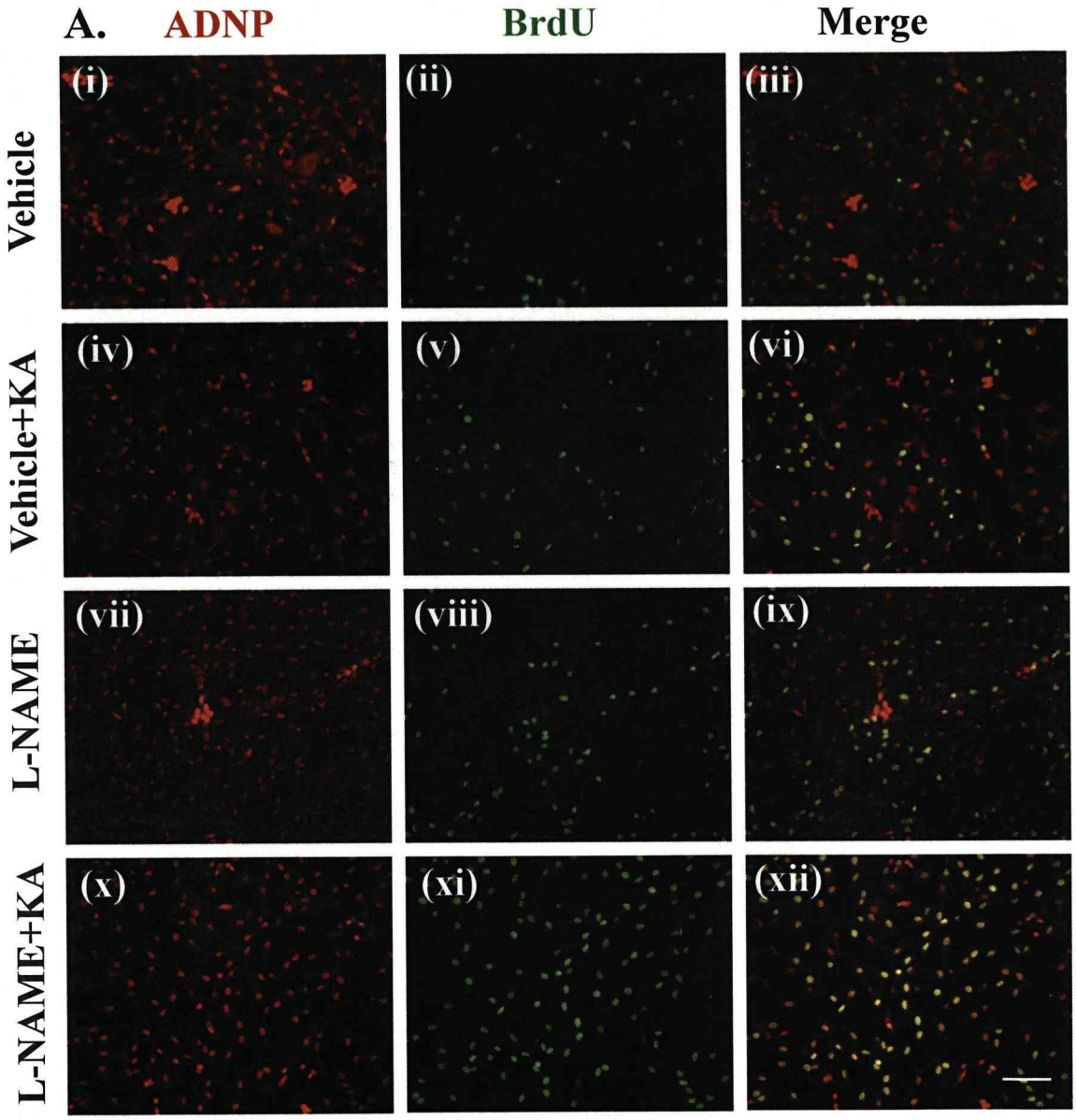


Figure 6.7 Light microscopy images show an increase in ADNP+ proliferating cells with NO inhibition following KA treatment 8DIV. Immunostaining with ADNP (red) and BrdU (green) revealed an increase in ADNP^{+} proliferating cells with L-NAME+KA treatment. Scale bar: 100μm.

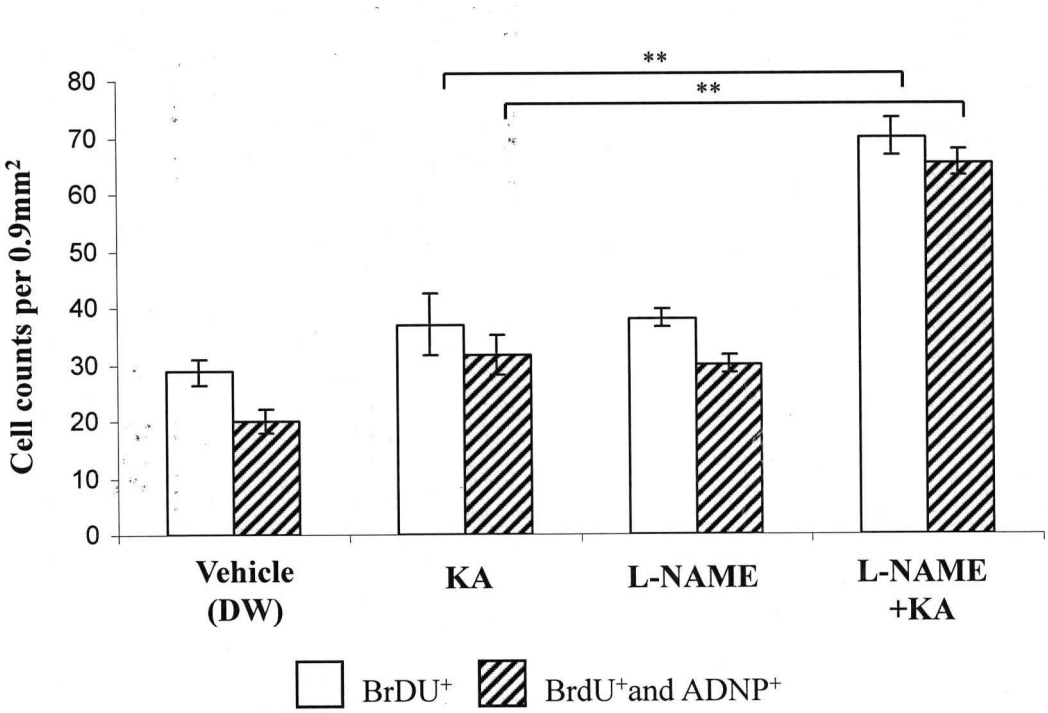


Figure 6.8 Histogram of cell counts to show an increase in ADNP$^+$ proliferating cells with NO inhibition following KA treatment 8DIV. Values are expressed as mean ± SEM. Significant differences are determined by one-way ANOVA with Bonferroni post-hoc test (*p <0.05, **p <0.01, ***p <0.001).

A.

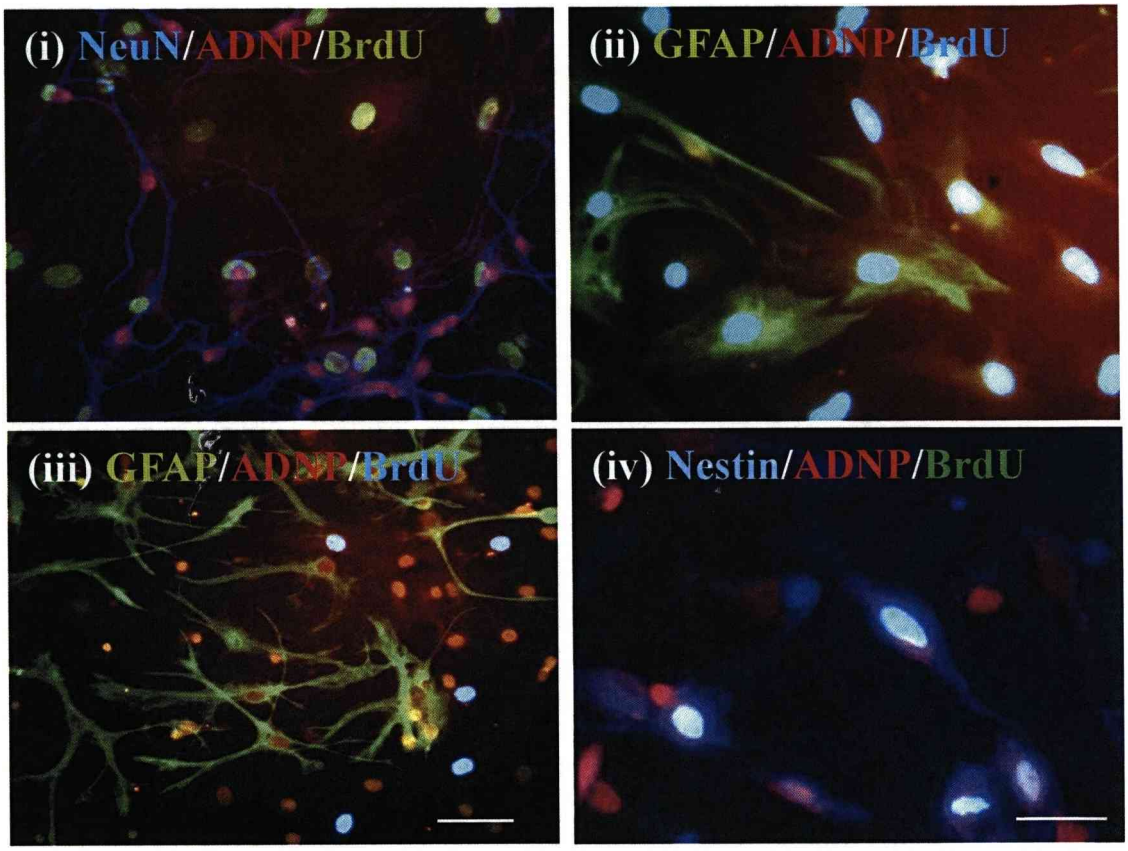


Figure 6.9 Photomicropgraphs of DG cells grown in vitro at 9 DIV (i) and (ii),for long pulse BrDU and 7 DIV (iii) and (iv) for short pulse BrDU. All cells were treated with L-NAME+KA and immunostained in (i) with NeuN (blue) ADNP (red), BrdU(green), in (ii) and (iii) GFAP (green), ADNP (red) and BrdU (blue). In (iv) cells were immunostained for Nestin (blue), ADNP (red), BrdU (green). Merged images show no merge with BrdU and NeuN, however, an increase in ADNP^{+} astrocytes with NO inhibition is shown following KA (iii), suggesting NO and ADNP are involved in the proliferation of precursor cells in vitro. Scale bar: 50μm in (i),(iii) and 20μm in (ii),(iv).

6.4 Discussion

One of the most recent neurophysiological roles of NO to be unveiled is its importance in the regulation of neurogenesis, although the proliferative roles of NO have already been described in that it reversibly inhibits cell division (Garg and Hassid, 1990) and inhibits cell proliferation in the adult mammalian brain (Packer et al., 2003). The dual role of NO in neuroprotection and neurotoxicity seems to extend to neurogenesis, as NO produced from nNOS appears to have antiproliferative effects (Park et al., 2002), whereas the induction of neurogenesis is attributed to the production of NO via iNOS or eNOS (Reif et al., 2004; Zhu et al., 2003). The isoform nNOS has been shown to be expressed by differentiated neurons surrounding the SVZ and also in neuronal precursors in the dentate gyrus (Moreno-López et al, 2000; Islam et al., 2003) suggesting the role of NO in neurogenesis. Moreover, it has been shown that the systemic administration of 7-Nitroindazole (7-NI), a relatively selective nNOS inhibitor, causes an increase in the number of mitotic cells in the SVZ, the olfactory bulb and the rostral migratory stream (RMS), however, this was not the case in the dentate gyrus (Park et al., 2002).

So far, the mechanisms via which NO regulates neurogenesis are largely unknown. In this chapter, I have used the general NOS inhibitor L-NAME with BrdU labelling in combination with immunocytochemistry in both in vivo and in vitro experiments to investigate the regulation of ADNP following KA-induced seizure. Results shown correlate with previous findings by Packer et al that NO negatively regulates adult neurogenesis, as an increase in $BrdU^+$ cells were observed following L-NAME administration (Fig. 6.1A(viii) and (Fig. 6.2). However, this was accompanied by a pattern of ectopic expression (Fig. 6.1A(viii) indicated by arrows) in the DG, which has not been previously reported. This may have been due to age or species differences. It may also be due to differences in L-NAME administration. In these experiments L-NAME was administered 3 times over a period of 24 hrs and so a more sustained inhibition of NO may lead to processes determining ectopic expression.

Previous findings have also shown that acute seizures augment neurogenesis and the migration of newly born neurons into ectopic regions such as the hilus and molecular layer (Parent et al., 1997; Bengzon et al., 1997; Gray et al., 1998). However, I did not observe such an effect following KA (Fig. 6.1A(v)). This may be because animals were administered diazepam after the initial seizure. Previous findings have employed animal models of TLE with prolonged seizures whereby an increase in the proliferation of granule cell progenitors is observed in the DG (Parent et al., 1997; Bengzon et al., 1997; Gray et al., 1998).

ADNP has been shown to play a role in development and neurogenesis, as ADNP knockout mice exhibit neural tube closure defects and death at E8.5-9.5 (Pinhasov et al., 2003). Furthermore, ADNP downregulation by small hairpin RNA technology causes a reduction in embryoid body formation and neurite numbers linking ADNP with neuronal differentiation and maturation (Mandel et al., 2008). My results show that NO inhibition causes a significant increase in the number of $BrdU^+$ and $ADNP^+$ BrdU cells following seizure or KA treatment (Fig. 6.1A(xii), Fig. 6.2, Fig. 6.7A(xii), Fig. 6.8), suggesting NO may regulate ADNP during proliferation, which may directly have an effect on cell proliferation or provide neurotrophic support to newly born cells.

NO is thought to play a role in most stages of neurogenesis, for example, via the growth factors involved in precursor proliferation e.g. BDNF, bFGF and EGF and NMDA receptor activation, which NO has been shown to regulate itself (Contestabile, 2000). During migration, a number of attractive and repulsive chemotropic factors such as ephrins, integrin subunits and reelin regulating migration may themselves be regulated by NO. In fact, recent studies using Reeler mutants demonstrated a down-regulation in nNOS protein expression in the olfactory bulb (Herrmann et al., 2007). The regulation of the molecular processes governing differentiation, integration and survival are thought to involve astrocytic signaling, and certainly, NO is known to regulate apoptosis and the survival of new cells (Kim et al 1999). Therefore, ADNP may well be involved in this astrocytic signaling, as an increase in the number of $ADNP^+$ astrocytes was observed

following L-NAME+KA treatment (Fig. 6.9A(iii)) compared to control treated (Fig. 6.6B(iv)).

ADNP is co-expressed with the PACAP receptor (PAC1R), a receptor involved in the release of gliotransmitters such as endozepines, which is regulated by PACAP,VIP, GABA and somatostatin (Masmoudi-Kouki et al., 2007). In the hippocampus, ADNP has been shown to co-immunostain with the neuron marker, neuron-specific enorase and all ADNP-immunopositive cells co-localize with PAC1R immunoreactivity. Interestingly, in the cerebral cortex, ADNP-immunoreactive cells co-immunostained with the astrocyte marker GFAP (Nakamachi et al., 2007). In 2003, Hüttmann et al showed an increase in proliferating radial glia-like astrocytes in the dentate gyrus after kainate-induced seizures, consistent with a recruitment of precursors for seizure-induced neurogenesis. It could be possible that during seizures, by regulating ADNP/VIP, NO causes astrocytes in the cortex/hippocampus to release these gliotransmitters.

CHAPTER 7

General Discussion

7.1 General Discussion & Future Directions

In vivo and in vitro experiments were carried out to investigate the role of the NO-cGMP pathway on ADNP/VIP expression following seizure/KA treatment. Animals were administered either L-NAME, a paradigmatic inhibitor of NOS, 7-NI a specific inhibitor of the neuronal isoform of NOS or ODQ which is a selective inhibitor of soluble guanylyl cyclase. This was to determine whether any changes in ADNP/VIP expression could be attributed to the direct effects of NO and more specifically via the neuronal isoform or via a cGMP mediated mechanism. Griess assays showed decreased levels of nitrite, the stable breakdown product of NO, in vivo with these compounds (Fig. 3.1). An increase in nitrite levels following seizure was also observed. This was accompanied by an increase in nNOS and iNOS expression in the hippocampus (Fig. 3.2), also consistent with other findings (Chuang et al., 2003; Zaja-Milatovic et al., 2008). When interpreting this effect of NO as anti-convulsant or pro-convulsant, caution must be applied as NO is previously reported to display both anti-convulsant (Penix et al., 1994, Kendrick et al., 1996; Gabriel et al., 2000; Sardo and Ferraro, 2007; Royes et al., 2007) and pro-convulsant effects (De Sarro et al., 1993; Tutka et al., 1996), depending on the species studied and epilepsy model employed. Such differences in findings on the effects of NO in epilepsy emphasizes the complexity of this molecule and factors such as NO concentrations, cell types, species and model must be taken into account.

In these studies, behavioural observations (Cosgrave et al., 2008) and other observations in our lab in mice show that NO inhibition with L-NAME advances the onset of the first full generalized seizure and that (50mg/kg) of L-NAME in mice can cause death. These results suggest NO to be an endogenous anti-convulsant in our model, however, as seizures were halted with diazepam administration after the initial seizure, we cannot conclude what the longer term effects of NO inhibition on seizure would be. NO cannot simply be categorized as anti- or pro-convulsant and the use of NO inhibitors/donors in the treatment of epilepsy may largely be dependent on concentration and timing. Further experiments, using highly specific inhibitors of each NOS isoform administered at

various time points could be used to determine the role each that isoform plays in the induction and maintenance of seizure.

As discussed in Chapters 3 to 6, it was shown that the NO-cGMP pathway is involved in the regulation of ADNP/VIP expression in the hippocampus/DG under both physiological and pathological conditions. One obvious conclusion to be drawn is that ADNP/VIP regulation is region-specific and that NO is regulating these proteins via different mechanisms in the DG and hippocampus. This may be due to the different cell types and the different roles each region plays in seizure activity. Patterns of immunostaining showed ADNP to be expressed in neurons and glia of the hippocampus, whilst VIP immunostaining was more glial-specific. ADNP is thought to be regulated by VIP (Giladi et al., 2007), however, a direct correlation between VIP and ADNP regulation in the hippocampus and DG was not always observed, suggesting other pathways and factors to be involved in their regulation. However, in the hippocampus it appears that NO inhibition causes an increase in both ADNP and VIP following seizure by 3 days. This was accompanied by a decrease in the number of $VIP^+/GFAP^+$ cells (and $ADNP^+/GFAP^+$ cells, but not quantified). Neuronal ADNP may, therefore, play a role in this region of the hippocampus after the onset of an initial seizure. As the cells of the CA1 and CA3 region are susceptible to cell death following KA-induced seizure, NO could serve to inhibit this ADNP neuronal regulation following seizure, leading to neuronal cell death. Hence, the initial rise in NO following seizure could be inhibiting the neuroprotective properties of VIP/ADNP.

Interestingly, the decrease in the number of $GFAP^+$ VIP and ADNP cells with NO inhibition following seizure also implies the regulation of glial cells via NO in the hippocampus following an initial seizure. Astrocyte and microglial activation occurs following seizure, a process involved in epileptogenesis, and studies in GFAP knockout mice showed hippocampal CA3 neurodegeneration following cerebral injury (Shapiro et al., 2008; Otani et al., 2006). GFAP knockout mice were also more susceptible to KA-induced seizures and had an increased number of pyknotic damaged CA3 neurons than WT mice (Otani et al., 2006). My findings suggest that an increase in NO following a

seizure is synonymous with an increase in glial activation in the hippocampus and hence NO inhibition causes a decrease in $GFAP^+$ cells in the hippocampus following seizure. These actions could pertain to the role of glial cells in neuroinflammation following seizures and the inverse relationship with VIP/ADNP infers NO regulated VIP/ADNP neuronal-glial signaling may be inhibited following a seizure, leading to the detrimental effects of neuroinflammation and neurotoxicity. In addition to the neuroinflammatory role of glial cells, another point for consideration in this area is that progenitors in the posterior SVZ migrate into the CA1 and CA3 fields and differentiate into glial cells (Parent et al., 2002). Could it be that NO augments glial cell differentiation in the CA1 and CA3 following a seizure? This could be a question for further investigation and analysis.

The distribution of these two proteins differed between the hippocampus and DG. VIP was found to be expressed at lower levels in the CA1 and CA3 regions than ADNP. However, in the DG a similar pattern of expression is seen between the two proteins, although one notable difference was a lack of VIP immunostaining in interneurons, despite previous findings to the contrary (Sloviter et al., 1987). However, I did see some co-immunostaining of VIP with nNOS in hilar neurons. Therefore, studies could be carried out to discern the interneuronal cell types involved in the regulation of VIP via the NO-cGMP pathway.

In the DG, NO inhibition caused an increase in ADNP expression by 3 hrs following seizure, this was accompanied by an increase in VIP mRNA expression by 3 hrs. By 3 days there was an increase in VIP^+ cells with NO inhibition following seizure suggesting a possible feedback mechanism/neuron-glial communication between $ADNP^+$ and VIP^+ cells in this region. As the DG is one of the few sites to display neurogenesis, BrdU labeling experiments were carried out to investigate if this increase in ADNP expression was related to neurogenesis. In vitro and in vivo experiments did confirm that NO is indeed involved in the regulation of cell proliferation following seizure. Previous work by Packer et al. (2003) corroborate these findings that NO negatively regulates mammalian adult neurogenesis. It could be argued then, that if there is an increase in NO following

seizure, this should attenuate neurogenesis which may seem contradictory to previous findings where animal models of TLE have shown prolonged seizures lead to an *increase* in the proliferation of granule cell progenitors in the DG (Parent et al., 1997; Bengzon et al., 1997; Gray et al., 1998). However, an explanation for this may lie in findings that reduced neurogenesis is associated with recurrent spontaneous seizures e.g. with chronic temporal lobe epilepsy in humans (Hattiangady et al., 2004). It is probable that the regulation of NO in neurogenesis, may like many of its other effects, be time and concentration dependent. I have shown that ADNP is involved in precursor cell proliferation and is regulated by NO following KA treatment both in vitro and in vivo. The antiproliferative properties of NO appear to depend on the inhibition by p53 of cyclin-dependent kinases and transcription factors by the Rb protein. However, the proliferative aspect of NO in the adult brain could be due to an increase in cGMP as demonstrated by pharmacological studies using sildenifil, an inhibitor of phosphodiesterase 5, leading to the accumulation of cGMP and causing an increase in neurogenesis (Gibbs, 2003; Zhang et al; 2003). cGMP is also thought to be involved modulating axonal guidance and neurite outgrowth of newly differentiated neurons (Hindley et al., 1997). Further BrdU-labelling experiments with sGC inhibitors could be carried out to determine if the cGMP pathway is involved in the regulation of neurogenesis and ADNP expression during seizure. Studies of SGZ cells in vitro have found that they retain the potential for self-renewal and the ability to differentiate into neurons, astrocytes and oligodendrocytes (Palmer et al., 1997). Hence, further experiments could determine the role of the NO and VIP/ADNP in cell differentiation in the DG and co-immunostaining with VIP and other cell markers could be carried out.

The actual mechanism via which NO interacts with the proteins ADNP and VIP cannot be determined from the work presented in this thesis and further investigations into how NO directly or indirectly modifies these proteins could be the subject for further investigation e.g. using the biotin switch assay to investigate post-translational modification via nitrosylation. The biotin switch assay converts nitrosylated cysteines to biotynylated cysteines which can then be detected by immunoblotting or avidin-affinity chromatography (Jaffrey & Snyder, 2001). In conjunction with NO donors, this technique

could be used to investigate whether ADNP or VIP are post-translationally modified via NO. Also, the human and mouse ADNP genes contain potential phosphorylation sites, which indicates the possibility of the protein being activated or deactivated through phosphorylation or dephosphorylation via cGMP produced via NO. Hence, proteomic and other protein phosphorylation detection methods could be used to further elucidate the mechanisms via which the NO-cGMP pathway regulates ADNP/VIP protein expression.

CHAPTER 8

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APPENDIX

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Regulation of activity-dependent neuroprotective protein (ADNP) by the NO-cGMP pathway in the hippocampus during kainic acid-induced seizure

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Activity-dependent neuroprotective protein (ADNP) is widely distributed in the cytoplasm of neurons and astrocytes of the hippocampus. Kainic acid (KA)-induced seizures increases neuronal nitric oxide synthase (nNOS) in neurons and inducible NOS (iNOS) in glia cells which coincides with a reduction in ADNP in the hippocampus. Inhibitors of NOS or soluble guanylyl cyclase (sGC) activity reduce ADNP under basal conditions in the absence of seizures. Treating animals with these inhibitors prior to KA-induced seizure, in particular, L-NAME (N^G-nitro-L-arginine methyl ester), advances the onset of the first seizure but reverses the loss of ADNP by 3 days after the first seizure. This suggests that the NO-cGMP pathway has a role in regulating ADNP under both basal physiological conditions and in the pathophysiological changes produced during epileptogenesis.

Keywords: Epilepsy; Kainic acid; Neuroprotection; ADNP; Nitric oxide synthase blockers; cGMP; sGC blocker

Introduction

Activity-dependent neuroprotective protein (ADNP) was first identified as a 'vasoactive intestinal peptide (VIP) responsive' gene product (Bassan et al., 1999; Gozes, 2007). The critical role of VIP in nervous system development and synapse formation suggested that ADNP may have similar important functions. ADNP contains the NAPVSIPQ (NAP) motif which is also found in another VIP-responsive protein and this peptide has been shown to have a neuroprotective function (Bassan et al., 1999; Zemylak et al., 2000; Gozes et al., 2003; Furman et al., 2004; Zaltzman et al., 2005). In a mouse model of head trauma, NAP protected neurons from death (Gozes et al 2005; Zaltzman et al., 2005). In another study using the hydrogen peroxide-induced stress model, ADNP reduced pro-apoptotic p53 protein in rat pheochromocytoma (PC12) cells (Steingart and Gozes, 2006). Importantly, a recent study by Vulih-Shultzman et al. (2007) demonstrated that ADNP$^{+/-}$ mice have cognitive deficits and increased phosphorylated tau, the hallmark of neurodegenerative diseases. ADNP has also been shown to be essential for brain formation and development (Pinhasov et al., 2003; Mandel et al., 2007), however, its role during postnatal pathophysiology of disease processes such as epilepsy has not been explored *in vivo*.

The kainic acid (KA)-induced animal model of epilepsy, that closely resembles human temporal lobe epilepsy (TLE) in some aspects, has been used to explore the effects of therapeutic interventions on epileptogenesis and seizure severity (Nadler, 1981; Cole et al., 2002; Velísek and Moshé, 2003; Scantlebury et al., 2007). Analysis of hippocampal pathology in human TLE has revealed neuronal loss and gliosis (Nadler et al., 1978; Ben-Ari, 1985; De Lanerolle et al., 2003) and, importantly, gene expression in TLE samples was consistent with increased glutamate release by astrocytes (Lee et al., 2007). Several factors contribute to cell death in KA-induced status epilepticus (SE), for example, excessive glutamate release and subsequent activation of *N*-methy-D-aspartate (NMDA) receptors and voltage gated calcium channels, and additionally

Abbreviations: ADNP, activity-dependent neuroprotective protein; AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; CA, Cornu Ammonis; cGMP, cyclic guanosine monophosphate; CREB, cAMP response element-binding protein; KA, kainic acid; L-NAME, N^G-nitro-L-arginine methyl ester; MAPK, mitogen-activated protein kinase; 7-NI, 7-nitroindazole; NMDA, *N*-methy-D-aspartate; ODQ, 1H-[1,2,4]oxadiazolo [4,3-a]quinoxalin-1-one; NO, nitric oxide; nNOS, neuronal NO synthase; PI_3K, phosphatidylinositol-3-kinase; qPCR, quantitative polymerase chain reaction; sGC, soluble guanylyl cyclase; SLM, stratum lacunosum moleculare; SP, stratum pyramidale; VIP, vasoactive intestinal peptide.

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suppression of GluR2, an α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor (Tomita et al., 2007; Friedman et al., 1994, 1998). All these factors lead to calcium-induced cell death that can be prevented if the animals are treated with diazepam prior to SE (Du et al., 1995).

The calcium influx activates neuronal nitric oxide synthase (nNOS) to produce NO (Garthwaite, 2005). The role of NO during epileptogenesis is controversial as it has been shown to have both anti-convulsant (Penix et al., 1994, Kendrick et al., 1996; Gabriel et al., 2000; Sardo and Ferraro, 2007; Royes et al., 2007) and pro-convulsant actions (De Sarro et al., 1993; Tutka et al., 1996), depending on the species studied and epilepsy model employed. NO is formed from L-arginine by any of the three NOS isoforms; neuronal NOS (nNOS), endothelial NOS (eNOS) or glial inducible NOS (iNOS). All three NOS isoforms are expressed during epilepsy. For example, eNOS is upregulated in a rodent model of SE within 3–24 h of intracranial injection of KA (Chuang et al., 2007; Liu et al., 2007), while nNOS and iNOS are upregulated in a mouse model of electrically-induced SE (Catania et al., 2003). NO activates soluble guanylyl cyclase (sGC) to generate cGMP which in turn activates protein kinase G (PKG) (Garthwaite, 2005). NO can also directly modulate the expression of transcription factors, for example, CREB mediates a cGMP/PKG-dependent anti-apoptotic signal cascade activated by NO (Nagai-Kusuhara et al., 2007; Zhuravliova et al., 2007; Riccio et al., 2006).

In a rodent model of epilepsy, VIP^+ neurons were spared in the hippocampus (Sloviter, 1987) and VIP receptor expression was shown to increase in human TLE (De Lanerolle et al., 1995). The nNOS knockout mouse has been shown to have fewer VIP^+ neurons in the brain (Kim et al., 2003) and it is therefore plausible that VIP-responsive ADNP production may have been reduced. Following on from these observations, as NO has been implicated in both neuroprotection at low concentrations and neurotoxicity at high concentrations (Calabrese et al., 2007), we hypothesised that there could be an interaction between NO production during epileptogenesis and ADNP synthesis. In order to explore this interaction we examined the effects on ADNP production in rats that were treated with NOS or sGC inhibitors prior to seizure induction with KA.

Materials and methods

Experiments were carried out on 35–40 day old male Wistar rats, which were kept under controlled environmental conditions (19–23 °C, 12 h light, 12 h dark) with food and water available *ad libitum*. All experiments were carried out under a UK Home Office project licence and had been considered by the University's Ethical Review committee.

Experimental design: drug treatment and kainic acid (KA) induction of seizure

The following drugs were used: NOS inhibitors, N^G-nitro-L-arginine methyl ester (L-NAME, 50 mg/kg i.p.) and 7-nitroindazole (7-NI, 50 mg/kg i.p.); the sGC inhibitor, 1H-[1,2,4] oxadiazolo [4,3-a]quinoxalin-1-one (ODQ, 10 mg/kg i.p.); and kainic acid (10 mg/kg i.p.). All chemicals were purchased from Tocris Cookson Ltd. UK, except 7-NI (Sigma, UK). KA and L-NAME were dissolved in distilled water (DW), and 7-NI and ODQ were dissolved in 20% DMSO. As appropriate, DW and 20% DMSO were used as vehicle controls. The effective drug doses were derived from previous studies (Gabriel et al., 2000; Bagetta et al., 2002; Catania et al., 2003; Gupta and Dettbarn, 2003; Kato et al., 2005; Thippeswamy et al., 2007a,b). In addition, a nitrite assay and/or cGMP immunoreactivity were carried out on samples of hippocampus to asses whether the NOS and sGC inhibitors were effective in blocking the NO and cGMP production, respectively.

Animal groups and treatments

In total, 120 animals were divided into 5 groups of 24 animals each. Each group received one of the following pre-treatments which were given over 24 h as three equally spaced doses: DW, 20% DMSO, L-NAME, 7-NI or ODQ. Subsequently, all animals were assigned to one of four groups (A to D) each consisting of 30 animals (six animals derived from each treatment group). Approximately 27 h after the first pre-treatment injection of drug or vehicle, groups C and D received KA (10 mg/kg i.p.), while groups A and B received DW (vehicle control for KA). Three hours later (i.e. 30 h after the first pre-treatment injection), groups A and C were euthanized and 3 days later groups B and D were euthanized. Group D animals were injected with diazepam (10 mg/kg, i.p.) after onset of the first full seizure (maximum of 3 h after receiving KA) to prevent development of SE which may lead to death. The amount of seizure activity the animals experienced was standardised as far as possible by permitting the animals to only experience a maximum of two seizures each lasting around 10–15 s, which represents a very mild insult. Although L-NAME pre-treated animals responded to KA earlier (25 ± 15 min, $n=6$) than the vehicle pre-treated group, the number and duration of seizure was still limited to two by administration of diazepam (3 day group) or euthanasia (3 h group). The 3 h (post-KA) time point was chosen because i) the onset of the first full seizure (see below) was variable, typically between 45 and 90 min. However, as described above, the number and duration of seizures during this period did not exceed two and all those animals that did not fall into this category were excluded from the experiment. ii) This allowed time for the KA to cross the blood-brain barrier and to initiate a chain of events following from direct receptor activation, through cell depolarisation, network activation, paroxysmal discharge, calcium influx to gene expression and protein synthesis prior to complete clearance or metabolism (for example, a review from Ben-Ari and Cossart, 2000).

Seizures were classified according to the Racine scale (Racine, 1972) using the following criteria: stage 1, freezing, facial twitching; stage 2, head nodding, salivation and arching of the back; stages 3–4, vigorous roaming around the cage, forelimb clonus, and rearing; and stage 5, rearing and falling followed by a series of muscle contractions. Tissues were only collected from those animals that showed full generalised seizures (tonic–clonic seizure) in the KA-treated groups C and D and extra animals were used to bring the group numbers back to 30/group. Further control experiments without KA were also carried out to confirm the direct effects of diazepam on nNOS, iNOS and ADNP production.

Half the animals from each group were deeply anaesthetised and fixed by vascular perfusion with 4% paraformaldehyde. Brain tissues harvested from these animals were processed for immunohistochemistry. The brains collected from the remaining were not fixed but were divided in half down the midline, one half of the hippocampus being used for RNA extraction and the other half for protein extraction.

RNA isolation

Experimental and control hippocampi were isolated and rapidly snap frozen in liquid nitrogen. Total RNA was extracted from each hippocampus using a Trizol–chloroform mixture (Gibco, UK), DNA and proteins were removed by salt-precipitation using isopropanol.

The RNA pellet was re-suspended in RNase-free water and each sample treated with RNase-free DNase to eliminate genomic DNA contamination (Promega, UK). The RNA concentration was determined with a Gene Meter spectrophotometer (ABgene, UK) and stored at −40 °C until further use.

Reverse transcription

The reverse transcription was carried out on 200 ng of total RNA for each sample using Invitrogen's SuperscriptII system according to the manufacturer's instructions. Initially, a 12 μl reaction mixture containing 25 μg/ml oligo dT primers and 0.5 mM dNTP incubated at 65 °C for 5 min, then the following were added to each sample: 4 μl of 5× First Strand Buffer [250 mM Tris–HCl (pH 8.3), 375 mM KCl, 15 mM $MgCl_2$], 10 mM DTT and 40 U RNaseOUT Recombinant Ribonuclease Inhibitor. Each reaction was mixed and then incubated at 42 °C for 2 min, 200 U of SuperscriptII was then added to each sample and incubated at 42 °C for a further 50 min. The reaction was then inactivated at 70 °C for 15 min, further held at 4 °C for 5 min and the cDNA was stored at −40 °C.

Semi-quantitative polymerase chain reaction

The polymerase chain reaction (PCR) was used for semi-quantitative analysis of ADNP, nNOS and iNOS transcripts. PCR was performed in an Eppendorf Mastercycler Gradient (Eppendorf, UK) thermal cycler. Appropriate primers were obtained from MWG Biotech, Germany, prepared as a 100 μM stock and stored at −40 °C. The following rat primer sequence was used: ADNP forward 5′-GGA CCA CAT TGT CAA TTC ACA CC-3′ and reverse primer 5′-GGA CAA GCG CTG CAG CAG AAA GG-3′; nNOS forward primer 5′-GAA CCC CCA AGA CCA TCC and reverse primer 5′-GGT TTG CTC CCA CTG TT-3′; iNOS forward primer 5′-AGC ATC ACC CCT GTG TTC CAC CC-3′ and reverse primer 5′-TGG GGC AGT CTC CAT TGC CA-3′; and β-actin forward 5′-ACG GTC AGG TCA TCA CTA TGG-3′and reverse primers 5′-AGC CAC CAA TCC ACA CAG-3′. A 50 μl reaction mixture was prepared in 0.2 ml capped thin walled tubes containing: 200 ng of cDNA template, 0.4 μl Taq DNA polymerase (Promega), 1.5 mM $MgCl_2$ (Promega), 0.2 mM dNTP (Invitrogen), 1 μl of each forward and reverse primer and 5 μl of 10× PCR buffer (Promega). PCR programmes were adapted from a standard protocol. As a control for the amount of cDNA used in the reactions, rat β-actin levels were analysed. PCR products from the RT-PCR reactions were electrophoresed on a 1% agarose gel, visualised with an Evenscan broadband dual wavelength transluminator in a MultiImageII Light Cabinet and digital images were captured using the AlphaImager unit (all equipments from Alpha Innotech Corporation, UK).

Quantitative polymerase chain reaction

Quantitative PCR was carried out using the Dynamo SYBR Green qPCR Kit (Finnzymes). Using an Opticon qPCR machine (GRI) a standard curve for each gene of interest or housekeeping gene was generated to determine copy numbers for unknown samples and a melting curve (65–95 °C with 0.2 °C increments) analysis was performed to confirm purity of products. Relative amounts of unknown samples were calculated from the amount of target gene and normalized to the amount of housekeeping gene (β-actin). Each experiment was carried out in triplicate and primer sequences were as described above. Thermal cycling conditions were as follows: 95 °C for 10 min, followed by 45 cycles of amplification at 94 °C for 10 s, 60 °C for 20 s, and 72 °C for 20 s followed by data acquisition. Data was analysed using Opticon Monitor 3.1 software (MJ Research, UK) and expression of target gene was normalized per 1000 copies of β-actin.

Western blotting

The hippocampi were dissected and homogenised in 500 μl RIPA buffer (50 mM Tris–HCl, 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS) containing a cocktail of protease inhibitors (Sigma, UK). Protein concentrations were determined using the BCA Protein Assay Kit (Pierce, USA). Proteins were resolved using the NuPAGE Novex 10% Bis–Tris gel system (Invitrogen, UK) and electrophoretically transferred to nitrocellulose membranes. Membranes were blocked with 10% donkey serum for 1 h, then incubated with primary antibodies (ADNP, 1:1000, Chemicon, UK; or β-actin 1:500, Sigma, UK) for a further 1 h at RT, washed with PBS (×3) and processed with secondary antibodies conjugated to a chromogen. After thorough washing with PBS (×3), membranes were visualised using the Westernbreeze chromogenic immunodetection system (Invitrogen, UK).

Immunohistochemistry

The animals were euthanized using halothane and perfused intracardially with 0.1 M phosphate buffered 4% paraformaldehyde (PBS-PFA). The brains were then dissected and further post-fixed in PBS-PFA overnight, cryo-preserved in 25% sucrose for a few days and frozen in liquid nitrogen cooled-isopentane prior to sectioning on a cryostat (Bright, UK). The brains were cut transversely into 15 μm sections and thaw mounted onto chrome-alum-gelatine coated slides (three sections/slide). Sections were washed in PBS to remove PFA and blocked with 10% donkey serum (DS) for 1 h at room temperature (RT). Double or triple immunostaining was carried out simultaneously on control and drug treated sections using the same reagents and antibodies. All antibodies were diluted in a diluting solution (PBS, 0.1% Triton X-100, 2.5% DS and 0.25% sodium azide). Sections were incubated overnight at 4 °C with the following primary antibodies in a combination of two or three depending on the species: ADNP (raised in rabbit, 1:500; Chemicon, UK), nNOS (raised in sheep, 1:1000, gift from Dr PC Emson, Cambridge, UK), GFAP (raised in rabbit/mouse 1:200; Chemicon, UK), iNOS (raised in rabbit 1:100; Chemicon, UK) and NeuN (raised in mouse 1:100; Chemicon, UK). The specificity of these primary antibodies are published elsewhere (Thippeswamy and Morris, 2001; Thippeswamy et al., 2007b). Primary antibody omission was routinely used as a negative control and a tissue section from a known positive control was also used in parallel (for example, axotomized dorsal root ganglion for nNOS and ADNP Thippeswamy and Morris, 2001; Thippeswamy et al., 2007b). On the following day sections were washed in PBS (×3) and appropriate anti-species antibodies, for example, donkey anti-rabbit Cy3 conjugated (1:300) and biotinylated donkey anti-sheep (1:500) were applied for 1 h at RT. All secondary antibodies are from Jackson Immunoresearch Laboratories, Inc., USA. After washing with PBS (×3), sections were treated with streptavidin–FITC (1:80, Vector Laboratories) for 1 h and then washed in PBS. For triple staining a third antibody was applied and sections were processed as above followed by a third fluorochrome, streptavidin–marina blue (1:50; Molecular Probes, USA) for 1 h. Sections were covered with VectaShield (Vector Laboratories), coverslipped and viewed with a

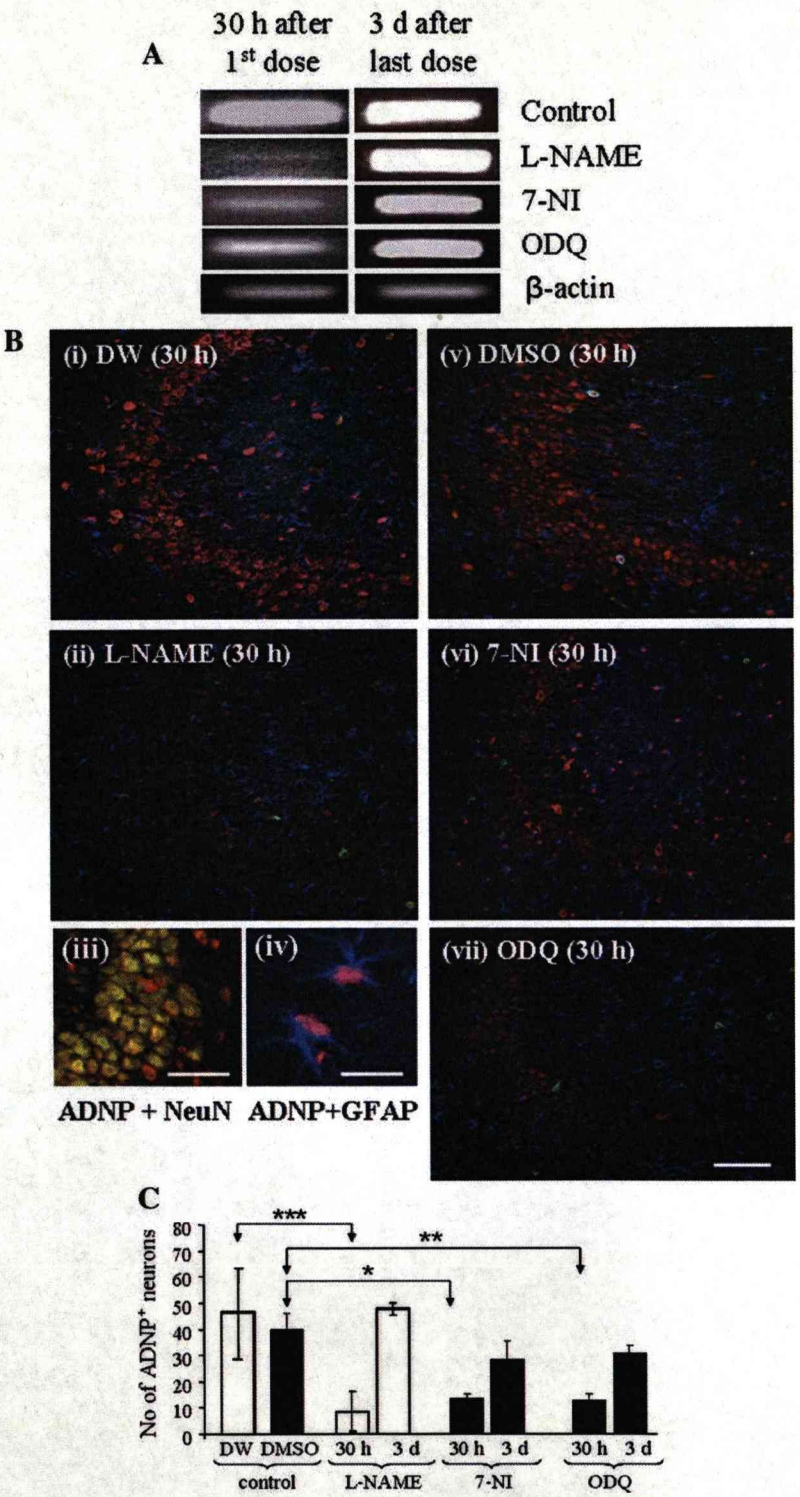


Fig. 1. Effect of NOS or sGC inhibition on ADNP in the hippocampus *in vivo*. (A) ADNP mRNA expression. L-NAME, 7-NI and ODQ reduced the amount of ADNP mRNA 30 h after the first dose of the drug compared with control. Three days after the end of drug treatment, ADNP mRNA levels returned to the pre-treatment control level. (B) Immunohistochemistry—effect of NOS or sGC inhibition (30 h) on $ADNP^+$ (red/pink in all), $nNOS^+$ (green except in iii) and $GFAP^+$ (blue in all) cells in the CA3 region. Control vehicle treatments (i) DW and (v) 20% DMSO had no effect on ADNP staining or distribution. Thirty hours after L-NAME treatment (ii) ADNP was markedly reduced in the CA3, with a smaller but similar reduction being seen after 7-NI (vi) and ODQ (vii) treatment. At higher magnification, ADNP can be seen to be co-localized in both neurons (iii, green cells are $NeuN^+$) and in astrocytes (iv, blue cells are $GFAP^+$). Scale bar: 100 μm (all) (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.). (C) Quantification of $ADNP^+$ neurons. By 30 h after the first dose of NOS or sGC inhibitor treatment, the number of $ADNP^+$ neurons significantly reduced compared with the appropriate control (*$p<0.05$, **$p<0.01$, ***$p<0.001$; $n=3$). By 3 days post treatment the number of $ADNP^+$ neurons had nearly returned to control values.

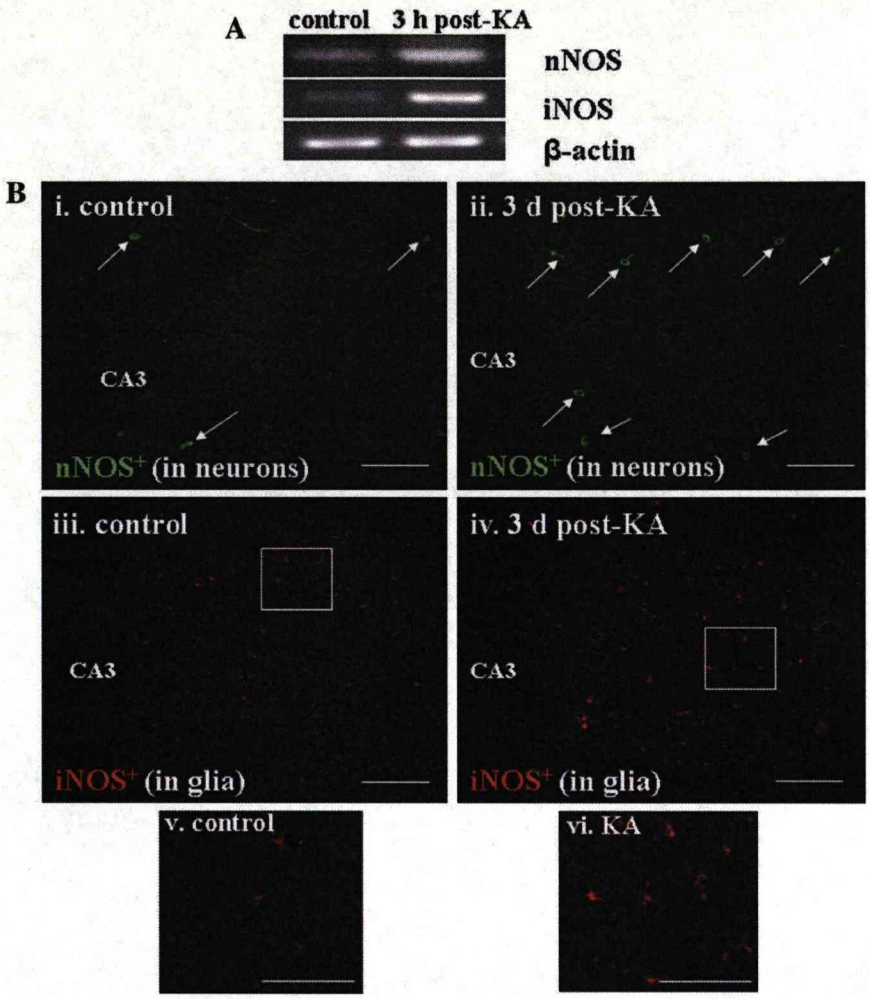


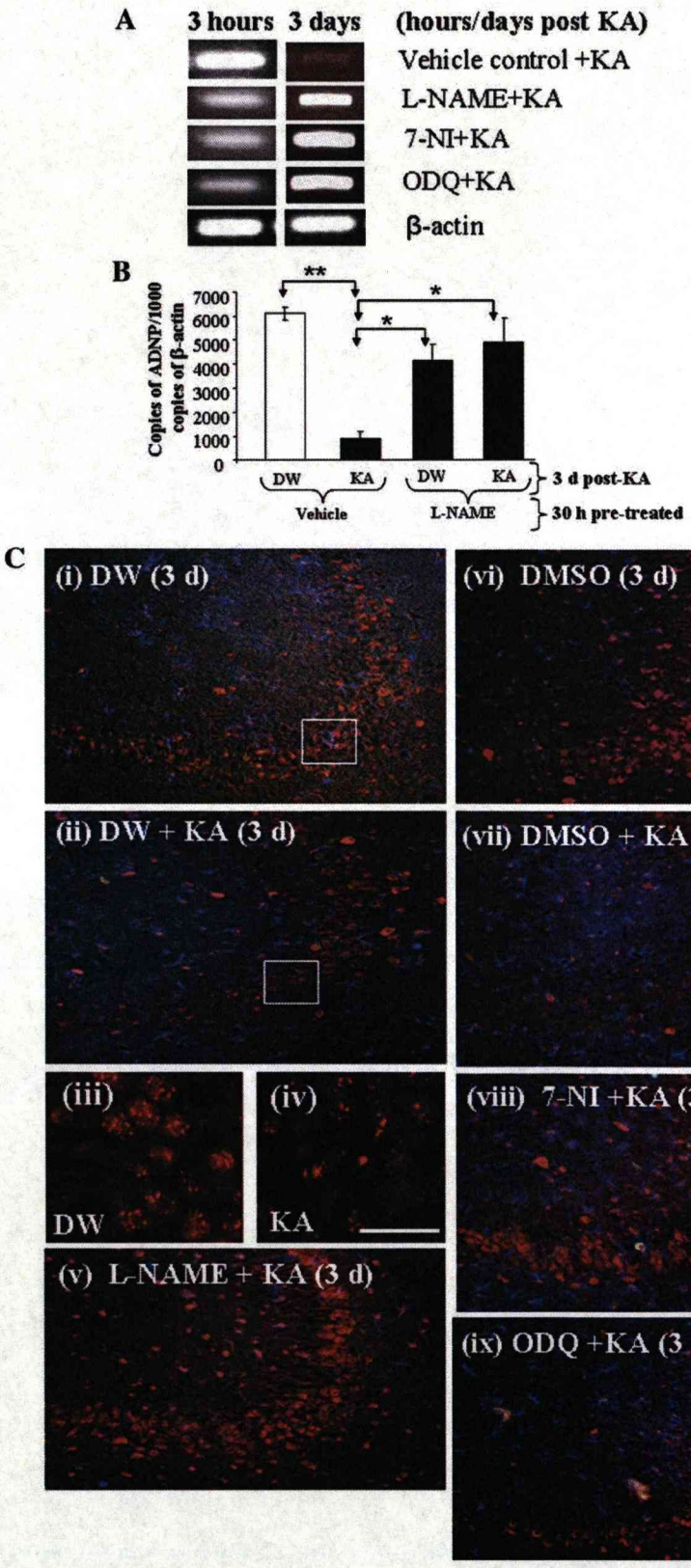
Fig. 2. Kainic acid (KA)-induced seizures increase NOS expression in the hippocampus. RT-PCR analysis (A) and immunocytochemistry (B) for nNOS and iNOS show that KA increases gene transcription by 3 h after KA treatment (A) and protein synthesis by 3 days, but not by 3 h (not shown). Green cells indicated by arrows in (i) and (ii) in 'B' represent $nNOS^+$ neurons and red cells in (iii–vi) are $iNOS^+$ (predominantly glia). (v) and (vi) represent higher magnification views of the $iNOS^+$ cells denoted by the box in (iii) and (iv) respectively. Note that there is an increase in $nNOS^+$ and $iNOS^+$ cells in KA-treated animal compared to the control. Scale bar: 100 μm (all). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Nikon inverted microscope (Nikon, UK) using appropriate wavelength filters for the fluorochrome. Images were captured by scanning sections using the appropriate filter for each fluorochrome and merged using IPL laboratory software (Nikon, UK).

Cell quantification

Immunopositive neurons were counted from the stratum pyramidale (SP) of CA1 ($NeuN^+$, $ADNP^+$) and CA3 ($ADNP^+$ only) regions of the hippocampus from animals receiving NOS or sGC inhibitors and/or KA to explore the effects of each treatment on ADNP synthesis. The average numbers (see below) were expressed as the percentage of $ADNP^+$ neurons against the total number of neurons ($NeuN^+$) in the CA1 region. In the CA3 region the average number of $ADNP^+$ neurons is presented instead of the percentage. This is because of the difficulty to count closely packed and overlapping neurons in the CA3 SP region compared to the CA1. The nNOS and iNOS immunopositive cell counts were made in the lacunosum moleculare (SLM) and stratum lucidum since $nNOS^+$ and $iNOS^+$ cells were predominant in these areas compared to other areas of the hippocampus, and moreover iNOS expression was inconspicuous in the stratum pyramidale of CA regions. Quantification of glial cells ($GFAP^+$) with respect to ADNP expression was not undertaken due to variability of GFAP expression in astrocytes as a result of the drug treatment.

Sections from approximately the same region of the hippocampus were selected for counting from control and drug treated animals which were processed for double/triple immunostaining simultaneously using the same reagents and/or antibodies. All slides were given a code and the code was revealed after cell count and statistical analysis. The appropriate wavelength filter for each fluorochrome was used to distinguish immunopositive cells from the rest and image captured, and then the filter was changed (without changing the field of view) to view a second or a third marker. Using IPL image analysis software, a threshold was set for each fluorochrome to distinguish positive versus negative staining and the same threshold was used for both drug and appropriate control. A minimum of three animals from each group were used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ODQ+KA
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7000
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(ii) DW + KA (3 d)
(vii) DMSO + KA (3 d)
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(viii) 7-NI +KA (3 d)
(v) L-NAME + KA (3 d)
(ix) ODQ +KA (3 d)

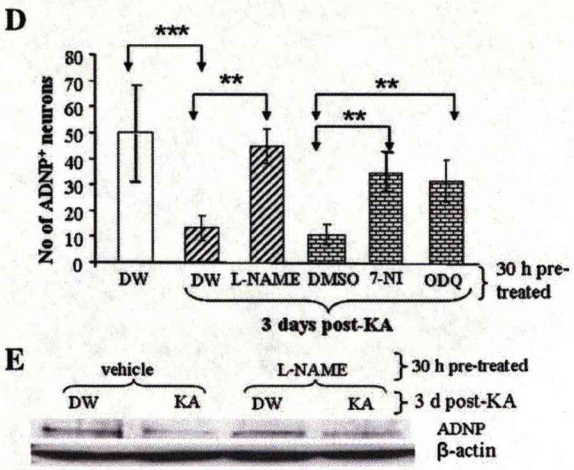

Fig. 3. KA-induced seizures markedly reduce ADNP mRNA (A and B) and protein as revealed by immunochemistry (C), cell counts (D) and Western blotting (E), 3 days after KA treatment (group D). Pre-treatment with the NOS blockers, L-NAME or 7-NI or the sGC blocker, ODQ reverses the effects of KA-induced ADNP suppression. (A, B) RT-PCR (A) and QPCR (B) analysis of ADNP mRNA revealed that KA suppresses ADNP mRNA by 3 days post-KA treatment, but not at the 3 h (A) time point tested. Treating with the NOS or sGC blockers for 30 h prior to KA reverses ADNP mRNA levels to nearly basal levels by 3 days. (C) Immunohistochemistry — appropriate vehicle (i, DW and vi, DMSO) treatments for both drugs (without KA) produced no reduction in ADNP staining, while KA treatment following these controls (ii, DW+KA, vii, DMSO+KA) produced a marked reduction in ADNP by 3 days. Pre-treatment with the NOS or the sGC blockers (v, L-NAME+KA, viii, 7NI+KA, ix, ODQ+KA) reversed this reduction. The higher magnification shows more detail of the marked reduction of ADNP in the soma of CA3 neurons after KA treatment (iv) compared with vehicle control (iii) (the areas correspond to the boxed regions in i and ii.). Scale bar: 100 μm (all). (D) Quantification of $ADNP^+$ neurons. KA treatment for 3 days significantly decreased the number of $ADNP^+$ neurons in the CA3 region compared with the appropriate control and pre-treatment with NOS or sGC blockers reverses KA-induced ADNP reduction (*$p<0.05$, **$p<0.01$, ***$p<0.001$; $n=3$). (E) Western blot analysis of ADNP from the whole hippocampus also reveals a reduction in the amount of ADNP protein following KA treatment which is partially reversed by pre-treatment with L-NAME.

for cell counting. The average cell numbers were calculated for each rat, the SEM calculated for each treatment group and p values obtained using ANOVA (Graphpad software Inc, USA) by comparing drug treated versus appropriate control. A 'p' value less than 0.05 was considered to be significant.

Results

Effect of NOS or sGC inhibition on ADNP expression under basal physiological conditions in the hippocampus

ADNP mRNA and protein was present in the hippocampus as revealed by RT-PCR and immunocytochemistry (Fig. 1). Cellular localization using cell-specific markers with double/triple immunostaining revealed that ADNP was present in both neurons and glia cells in the CA1 and CA3 regions of the hippocampus (Fig. 1B). Interestingly, apical dendrites in the stratum oriens (SO) of the CA1 pyramidal neurons were intensely stained for ADNP (Fig. 4A), while mossy fibres in the SLM were not stained (not shown). Several pyramidal cells in the CA1 and CA3 and interneurons and astrocytes in the SO, stratum radiatum (SR) and stratum lucidum (SL) also contained ADNP in their cell bodies (Figs. 1B, 4A).

In order to explore whether the NO-cGMP pathway has a role in the physiological expression of ADNP under control conditions in the hippocampus, animals treated with the NOS or sGC inhibitor (without the induction of seizure with KA, groups A and B) were examined. ADNP mRNA expression was reduced by L-NAME, 7NI or ODQ compared to controls (DW or DMSO) in group A (control group for 3 h post-KA group C, euthanized 30 h after the first drug dose) but not in group B (control group for 3 days post-KA group D, euthanized 3 days after the last drug dose) (Fig. 1). This shows that after a transient block of the NO-cGMP signalling system, ADNP production is inhibited but recovers to control levels within 3 days.

The effect of NOS or sGC inhibition on the topographic distribution of ADNP containing neurons in the CA3 and CA1 regions of the hippocampus was further examined by immunostaining (Fig. 1B). Both nitrite and cGMP immunoreactivity of the hippocampus from the NOS and sGC inhibitor-treated animals decreased in group A (30 h after the first dose) but had recovered in group B (3 days after the last dose) (data not shown). In group A animals, the number of $ADNP^+$ neurons in the CA3 region of the hippocampus was significantly reduced in the L-NAME treated group compared to the vehicle control (Figs. 1B, C). Similarly, 7-NI or ODQ treatment also caused a reduction in the number of $ADNP^+$ neurons in the CA3 as compared to the DMSO control group (Figs. 1B, C). However, 3 days post drug treatment (group B) there was no significant difference in $ADNP^+$ neuron numbers compared with appropriate vehicle control groups (Fig. 1C). Similar observations were made from the CA1 region (data not shown).

Kainic acid treatment increases nNOS and iNOS mRNA and protein synthesis in the hippocampus

PCR analysis of mRNA for nNOS and iNOS revealed a significant increase in their expression in the hippocampus of KA-treated animals (3 h post-KA, group C), compared to the vehicle control (Fig. 2A), but increased protein synthesis occurred later. Immunostaining of the 3 days post-KA hippocampi (group D) for nNOS and iNOS protein revealed an increase in the number of $nNOS^+$ cells (predominantly neurons) and $iNOS^+$ cells (predomi-

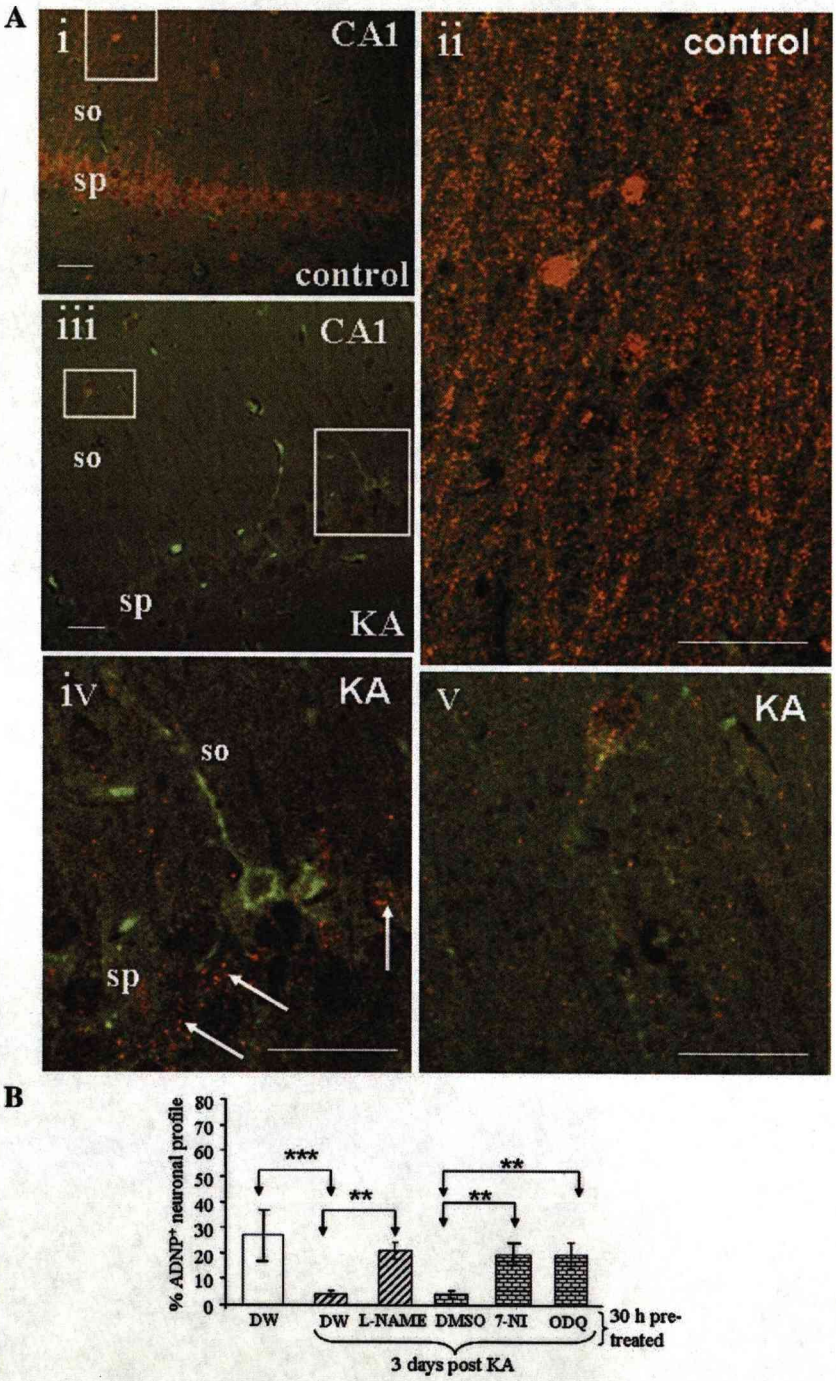

Fig. 4. Effect of KA and NOS or sGC inhibition on ADNP in CA1 region of the hippocampus *in vivo*. (A) Immunohistochemistry. (i) In control vehicle treated staining for ADNP is seen in the cell bodies of CA1 pyramidal neurons in the stratum pyramidale (sp). (ii) Higher magnification of the area shown by the box in (i), shows intense ADNP staining in apical dendrites of the pyramidal neurons traversing the stratum oriens (so). (iii) KA-induced seizure increases the number of $nNOS^+$ cells (green) while dramatically reducing $ADNP^+$ (red) in the pyramidal cells of CA1 region of the hippocampus. iv. Increased nNOS is primarily in the soma and dendrites of neurons, while the residual ADNP is confined mainly to the nucleus of a few neurons in the stratum pyramidale (sp). (v) Higher magnification of the area indicated by the box in iii (in the 'so' region) shows the loss of ADNP staining from the apical dendrites and reveals nNOS staining in some fibres. Scale bar: 100 μm (all). (B) $ADNP^+$ neurons quantification. The percentage of $ADNP^+$ neurons were derived from counting all the neurons that were identified by NeuN staining, and ADNP positive neurons from the CA1 stratum pyramidale. In controls, approximately 30% of neurons expressed ADNP and this was markedly reduced by 3 days after KA treatment. Almost complete reversal was seen with the NOS or sGC blockers. $^*p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$; $n=3$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

nantly microglia and astrocytes) compared with the control (Fig. 2B). For example, 3 days post-KA treatment, in the SL and SLM together, in control animals there were 5±3 $nNOS^+$ and 9±4 $iNOS^+$ cells, and this increased to 22±7 and 38±11 cells, respectively. The SL and the SLM had greater numbers of nNOS and iNOS cells (Fig. 2B) compared to other areas of the hippocampus. In addition, in 3 days post-KA treatment there was increased nNOS staining in the neurites, whose cell bodies were located in the outer margins of the SP (Fig. 4B) in the CA3 and CA1 regions suggesting an increase in the local release of NO. The number of $nNOS^+$ and $iNOS^+$ cells did not change in control animals treated with diazepam (without KA).

Effect of NOS or sGC inhibition prior to kainic acid treatment on ADNP in the hippocampus

The KA treatment caused a marked reduction of both ADNP mRNA and protein synthesis in the 3 days post-KA animals (group D) as revealed by RT-PCR/qPCR, immunocytochemistry and Western blot analysis (Fig. 3). In haematoxylin and eosin, and TUNEL stained sections of the hippocampus from both 3 h (group C) and 3 day (group D) post-KA animals did not reveal any abnormal morphology or cell death (data not shown) indicating that the reduction in ADNP is not due to cell loss. Since group D animals received diazepam to prevent status epilepticus and subsequent cell death in the CA regions, it might be argued that the reduction in ADNP was in some way due to this treatment. However, control experiments (without KA) to check this possibility revealed that diazepam alone had no effect on ADNP levels (data not shown).

The suppression of ADNP mRNA by KA alone (without the NOS or sGC inhibitor pre-treatment) was evident in the 3 day post-KA-treated animals (group D) but not in the 3 h post-KA group (group C). However, L-NAME, 7-NI or ODQ pre-treatment reduced ADNP mRNA levels in the 3 h post-KA group C, compared to appropriate vehicle pre-treatment groups within this group (vehicle +KA) (Figs. 3A, C, D). This implies that this initial reduction in ADNP mRNA levels was due to reduced production of NO and cGMP as opposed to the effects of KA (for 3 h), which correlates with the effects of these drugs seen under basal conditions (Fig. 1). Interestingly, by 3 days post-KA treatment (group D), ADNP reduction was reversed in the hippocampus in the NOS or sGC inhibitor pre-treated group but not in the vehicle pre-treated groups (Fig. 3). This indicates that increased NO levels, as evident from greater numbers of $nNOS^+$ neurons and $iNOS^+$ glia cells (Fig. 2B) following KA treatment (by 3 days), decreases ADNP. Immunocytochemical analysis of the CA3 and CA1 regions of the 3 day post-KA-treated animals (group D) for ADNP suggested that a reduction of ADNP synthesis was evident in both neurons (Figs. 3C, 4A) and astrocytes (Fig. 3C). In the control group ADNP was observed throughout the soma (SP) and apical dendrites of the CA1 pyramidal neurons (SO), while in the 3 day post-KA treatment group ADNP staining was observed in clusters and/or in punctate form within the nucleus of a few neurons in the SP. ADNP was almost completely suppressed in the apical dendrites in the SO (Fig. 4A).

Effect of NOS or sGC inhibition prior to kainic acid treatment on the onset of the first seizure

Following KA administration, animals were monitored and the symptoms leading to the onset of the first full tonic–clonic (generalised) seizure were scored according to Racine's seizure severity scale (stages 1–5) as described previously. In the KA-treated group, without the NOS or sGC blocker pre-treatment, animals progressed steadily from stages 1 to 5 within 90 min of KA treatment. In L-NAME pre-treated animals there was no significant difference in the symptoms at each stage leading up to the onset of the first generalised seizure (stage 5), but the duration of symptoms at each stage from 1 to 4 decreased. However, the duration of stage-5 symptoms did not exceed 10–15 s in all KA-treated groups. In general, L-NAME pre-treated animals exhibited the first full tonic–clonic seizure sooner (25±15 min) than animals that had been pre-treated with the vehicle control (DW or DMSO), 7-NI or ODQ (70±20 min), and the number of stage-5 seizures did not exceed more than two in any group (euthanized by 3 h without diazepam or seizures were terminated by treating them with diazepam in 3 day post-KA group). This may suggest that the non-specific block of all NOS isoforms by L-NAME, somehow, facilitates KA-induced early onset of seizure activity while more selective block does not. It is speculated that the decreased ADNP by L-NAME treatment prior to KA may be one reason for early onset of the first seizure.

Discussion

A neuroprotective role for VIP-responsive ADNP and its derivative peptides, such as NAP, has been identified (Gozes, 2007). Although studies of ADNP in experimental epilepsy have not been reported VIP does seem to have a role. VIP levels fall acutely in experimentally induced seizures (Romualdi et al., 1992), while post-mortem hippocampus samples from human TLE have increased VIP binding (De Lanerolle et al., 1995). Initial studies of ADNP suggested that it was primarily located in glia cells (Gozes, 2007). Previously, Bassan et al. (1999) showed increased basal levels of ADNP mRNA in astrocytes in response to VIP and, interestingly, VIP and/or its derivative peptides induce NO and cGMP production in cortical cultures (Ashur-Fabian et al., 2001). We have previously demonstrated an important role for NO in neuron–glia communication and neuroprotection (Thippeswamy et al., 2005, 2007a). It is thus plausible that NO could be involved in regulating VIP-responsive ADNP function. In the present study ADNP was detectable under basal conditions in the pyramidal neurons and interneurons of the hippocampus in addition to astrocytes. The antibodies used were raised against the peptide sequence 989–1015 of the human ADNP which is identical in rat and mouse, and does not share homology with other proteins when used in a BLAST search. Interestingly, ADNP staining in the apical dendrites of the pyramidal cells in the SO was intense under basal conditions, suggesting large amounts of the protein (Fig. 4A), but was conspicuously absent in the SL and mossy fibres. This reveals a highly selective distribution indicating a cell-specific as opposed to a generalised function.

Experimental-induced seizures provide a simple model for producing neuronal stress leading to neuronal death. Several methods have been employed to induce seizures (Nadler, 1981; Cole et al., 2002; Velísek and Moshé, 2003; Scantlebury et al., 2007) and for the present study we used KA. At the doses used in these experiments, KA initially activates kainate receptors in the hippocampus (Ben-Ari and Cossart, 2000) but this rapidly leads to more widespread neuronal depolarisation and release of transmitters such as glutamate acting on both AMPA and NMDA receptors. The neuron depolarisation and NMDA channel activation leads to calcium influx resulting in activation of NOS and subsequent NO production (Kato et al., 2005). NO released from nNOS activates

sGC to produce cGMP in many areas of the brain including the hippocampus (Garthwaite et al., 1989; Teunissen et al., 2001). To explore the effects of NO and cGMP on ADNP distribution and synthesis we examined its distribution in the presence and absence of NOS and sGC inhibitors. The sGC inhibitor, ODQ was used to block cGMP production to understand whether ADNP is also regulated by the sGC-cGMP pathway. Several others have used these NOS or sGC blockers, *in vivo*, via the intraperitoneal route and have demonstrated that they cross the blood-brain barrier (Bagetta et al., 2002; Catania et al., 2003; Gupta and Dettbarn, 2003; Kato et al., 2005; Chuang et al., 2007; Liu et al., 2007; Parathath et al., 2007). The decreased nitrite content and lack of cGMP immunoreactivity in the hippocampus of animals treated with NOS or sGC inhibitor confirmed that the drugs were effective in blocking NO and cGMP production.

The KA treatment dramatically reduced ADNP mRNA and protein synthesis by 3 days post-KA treatment but not at earlier time points examined (3 h and 24 h post-KA, data not shown for 24 h) suggesting a complex pathway involved in KA-induced ADNP suppression. The morphological analysis together with TUNEL staining for apoptosis in the hippocampus from these animals confirmed that there was no cell loss. The delay in suppression of ADNP synthesis coupled with the later widespread loss of ADNP staining in all the CA regions studied could result from increased NO production by KA-induced seizures. The 3 day post-KA group was treated with diazepam before onset of SE to prevent neuronal death. It has been previously demonstrated by Du et al. (1995) that diazepam rescues neurons from calcium-induced death in a KA model of epilepsy. Therefore, the decrease in ADNP mRNA and protein in KA-treated animals is not due to cell loss. Diazepam limits the severity of seizures (Pitkänen et al., 2005) and this would be predicted in turn to reduce excessive NO production, thus reducing the neurotoxic actions of NO (Rajasekaran, 2005; Chuang et al., 2007). In the present study, the animals that received diazepam and the NOS or sGC blocker showed complete reversal of the ADNP loss 3 days after KA treatment. These results suggest that the use of antiepileptic drugs combined with NOS inhibition may be beneficial (Paul, 2003; Luszczki et al., 2006).

The precise mechanism of KA-mediated ADNP suppression by NO will require much more detailed research to elucidate. However, following KA treatment it was noted that ADNP was mainly located in the nuclei of the pyramidal neurons while in the animals pre-treated with the NOS blocker, it was distributed throughout the soma and dendrites. As ADNP is synthesised in the cytoplasm, the presence of peptide in the nucleus is indicative of translocation and it is possible that ADNP may function as a DNA-binding protein/transcription factor to regulate gene expression. Zemlyak et al. (2007) have recently shown that the ADNP-derivative, NAP protects KA-treated hippocampal neurons in culture by interacting with microtubule-associated protein (MAP). ADNP and NAP can also modulate polyADP-ribosylation to promote neuronal differentiation and survival via MAPK-PI_3K/Akt pathways (Mandel et al., 2007; Mandel and Gozes 2007; Pascual and Guerri, 2007), similar to the effect caused by NGF on PC12 cells (Visochek et al., 2005).

Increased NO concentration in the CNS causes DNA damage, altered mitochondrial membrane potential and activation of polyADP-ribosylation leading to neuronal death (Zhang et al., 1994; Dawson and Dawson, 1995; Wallis et al., 1996). Depending on the NO levels, it can modulate DNA-binding activities of the cAMP response element-binding protein (CREB, a transcription factor) to mediate the cGMP-PKG-dependent anti-apoptotic signal induced by NO (Nagai-Kusuhara et al., 2007; Zhuravliova et al., 2007; Riccio et al., 2006). Interestingly another VIP-derived peptide, ADNF-9, also protects neurons from iNOS-mediated toxicity in the hippocampus in a hypoxic-ischemia model (Kumral et al., 2006). Low concentrations of NO found under basal conditions are known to be beneficial (Contestabile and Ciani, 2004; Thippeswamy et al., 2006; Calabrese et al., 2007). The reduction of ADNP produced by blockade of NOS or sGC (in the absence of seizure) suggests that the basal production of NO may be important for maintaining ADNP synthesis in the hippocampus. Based on these observations it is plausible that ADNP and NO may interact to regulate neuronal survival. Recent studies have shown that NO interacts with ADNP downstream targets. Stroissnigg et al. (2007) have demonstrated that MAP can undergo *S*-nitrosylation which is another action of NO and this is involved in the regulation of growth cones morphology. In the present study, KA-induced NO suppression of ADNP in apical dendrites of the pyramidal neurons may also suggest an NO–ADNP interaction in orchestrating the onset of seizure during epileptogenesis.

In summary, under physiological conditions, a low concentration of NO appears to promote ADNP synthesis since the block of NO decreases ADNP and it advances the onset of the first seizure in the KA-induced epilepsy model. KA increases NO production as evident from greater numbers of $nNOS^+$ and $iNOS^+$ cells in the hippocampus and, interestingly, this high level of NO suppresses ADNP. Treating animals with the NOS or sGC inhibitor prior to KA reverses ADNP suppression, by 3 days post-KA treatment, implying its regulation by the NO–cGMP pathway.

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